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13. ABSTRACT (Maximum 200 Words)

This research had as its goal inhibiting the expression of two pathways critical to prostate cancer progression, one mediated by the tyrosine kinase receptor c-Met, and the second mediated by urokinase plasminogen activator (uPA). Each pathway has been demonstrated to be important to prostate tumor progression, and further, they may be related, i.e., increased c-Met expression leads directly to increased uPa expression. The purpose of this research was to determine the relationship between uPa and c-Met expression and prostate tumor progression. By using various strategies to inhibit these molecules, the second purpose was to determine if decreased tumor growth and/or tumor progression would result. Thus, the scope of the work utilized human prostate tumor cell lines, and examine in vitro and in vivo the effects of in habiting these pathways. We have demonstrated that inhibition of c-Met results in inhibition of growth at the primary tumor site and significantly, prevents development of lymph node metastases in an orthotopic nude mouse model. Inhibition of uPaR also inhibits metastatic growth. UPaR expression increases in clinical specimens of more progressed disease. Thus, c-Met and uPAR contribute to CaP progression and further development of inhibitors for these enzymes is promising for CaP treatment.

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Table of Contents

INTRODUCTION

DOD Award number DAMD17-00-1-0524, "Cellular Mechanisms Regulating Urokinase-Type Plasminogen Activator in Hormone Refractory Prostate Cancer: A Novel Therapeutic Target" had as its goal inhibiting the expression of two pathways critical to prostate cancer progression, one mediated by the tyrosine kinase receptor c-Met, and the second mediated by urokinase plasminogen activator (uPA). Each pathway has been demonstrated to be important to prostate tumor progression, and further, they may be related, i.e., increased c-Met expression leads directly to increased uPa expression. The purpose of this research was to determine the relationship between uPa and c-Met expression and prostate tumor progression. By using various strategies to inhibit these molecules, the second purpose was to determine if decreased tumor growth and/or tumor progression would result. Thus, the scope of the work utilized human prostate tumor cell lines, and examine in vitro and in vivo the effects of in habiting these pathways. Finally, the studies examined expression in human tissue specimens. DAMD17-00-1-0524 was originally awarded to Robert Radinsky, Ph.D. with the award date of July 1, 2000. Dr. Radinsky subsequently left the University of Texas M.D. Anderson Cancer Center for Amgen Pharmaceuticals and I (Gary E. Gallick, Ph.D.) was approved to become the Principal Investigator of this grant. A thorough review of the project was undertaken when I was named PI, new animal protocols were written, in response to concerns of the U.S. Army veterinarians; thus the project was initiated only four months prior to the submission of the "year 1" progress report. As a result, I requested and received a one-year no cost extension on this Award. With this extension, we have essentially successfully completed the tasks originally proposed. Specifically, we have demonstrated that the proposed strategies to inhibit both c-Met and uPaR (as described in the Tasks) were successful in inhibiting the growth of human prostate tumor cells in orthotopic nude mouse models. These studies have important implications for novel therapeutic approaches to therapeutic strategies for human CaP. Further, completion of Tasks 1 and two have resulted in three publications (Davies et al. (2002); Boyd et al. (2003); Kim et al. (2003))-see APPENDIX. The studies were the first to directly demonstrate the efficacy of targeting c-Met in a prostate cancer model, generating sufficient excitement in the field for the Kim et al. (2003) paper to be featured in Clinical Cancer Research, and the subject of a commentary by Shinomiya N and Vande Woude GF, 2003. As completion of the Tasks has resulted in these publications, the Final Report will summarize our accomplishments; more complete details may be found in the manuscripts in the APPENDIX.

BODY

Task 1

The major thrust of task 1 involved evaluation of high and low metastatic prostate cancer cells to invade a three dimensional matrix after HGF treatment and in the presence of uPa antagonists and c-Met ribozymes. The goal of these studies was to determine if inhibition of uPa/uPaR and c-Met alone was effective in reducing in vitro migration and invasion, and if inhibiting both uPa-mediated and c-Met-mediated signaling was more effective than inhibiting either alones. Using a modified Boyden chamber assay in which cells migrate or invade through pores in the presence or absence of inhibitor, first, the ability to inhibit migration was determined, then the ability to

invade through matrigel was determined. The approach to this task is almost identical to that outlined in the original proposal.

Migration

Migration does not require a three dimensional matrix, but is one of the key properties of metastasis. First, we tested the ability of c-Met inhibited cells to migrate in a Boyden chamber assay. For these experiments, the highly migratory, highly invasive PC3-LN4 cells were used. Cells were mock-infected, infected with Ad-c-Met (harboring the c-Met ribozyme) or Ad-PU-1 (a control adenovirus with all the sequences except the ribozyme-Abounader et al., 2002). As presented in Kim et al., Figure 3 (see APPENDIX) At a multiplicity of infection of 25, complete inhibition of migration was observed with Ad-c-Met, with no inhibition observed with control virus. These results demonstrated that inhibition of c-Met effectively blocked migration.

Invasion

Invasion of cells was examined through the use of a Boyden chamber on which the surface on which cells are grown was coated with Matrigel; thus cells must degrade the equivalent of a basement membrane. Using the same procedure as that described for migration assays, we demonstrated that inhibition of c-Met completely inhibited invasion through Matrigel (Kim et al., Fig 3-see APPENDIX). Similar invasion assays were performed with A6, a small peptides affecting uPAR function (Guo et al., 2000). These results are now also published (Boyd et al, 2003-see Appendix Fig. 2). While not as effective as inhibition of c-Met, A6 inhibited invasion of PC3-LN4 cells in a dose-dependent manner. To ensure that decreased invasion did not result from decreased proliferation (hence fewer cells to invade), a growth curve was performed in the presence of A6. A6 did not affect proliferation at concentrations at which invasion was inhibited. Thus, results from Task 1 demonstrate that inhibition of either uPAR or c-Met inhibits cellular invasion. Because of the complete effectiveness of c-Met inhibition in blocking migration and invasion, we did not examine the combination of the two inhibitors.

Task 2

Task 2 focused on determining if inhibition of uPAR and c-Met effects growth and progression of prostate tumor cells in an orthotopic nude mouse model. For these experiments, PC3-LN4 (highly metastatic) prostate tumor cells were implanted into the prostates of nude mice, and "treatment" regimens were performed by use of uPAR antagonist (A6) or an Adnovirus expressing a c-Met ribozyme, exactly as proposed in the initial application. As indicated in the introduction, both strategies were successful, and have led to publications (see APPENDIX).

Inhibition of uPAR

For this study, A6 was injected daily into tumor-bearing mice. Mice were sacrificed when signs of morbidity were evident, in accord with the guidelines of the Institutional Animal Care and Use Committee and those in response to the DOD veterinary staff. Results of these experiments are shown in Figure 1 (Boyd et al., Figure 3 in APPENDIX).

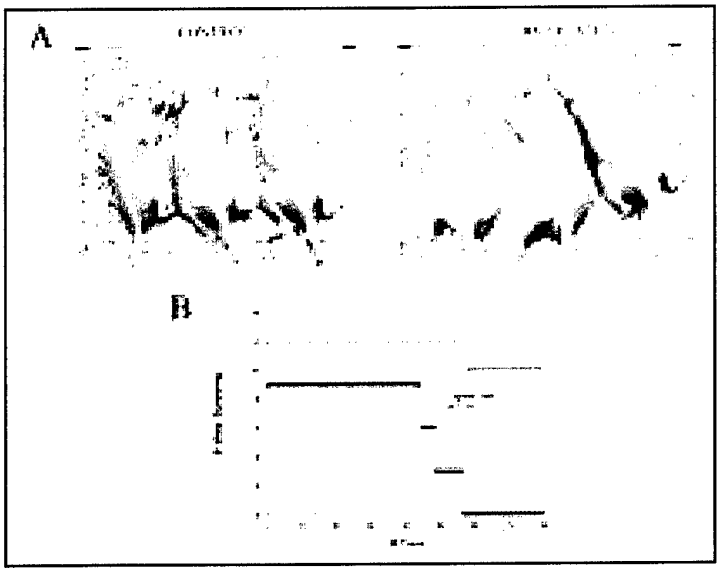

Figure 1. Treatment of tumor-bearing mice with the uPAR antagonist, A6. A; Pictures of mice bearing PC3-LN4 tumors after daily treatment with carrier PBS control) or 27mg/Kg A6. B. Mice were orthotopically injected with 2 million cells. When primary tumors were palpable, mice were divided into two groups with one receiving PBS alone and the other administered with 75 mg/KG A 6 on a daily basis. Mice were sacrificed when moribund as determined by standard animal use and care criteria. Differences in survival rates were statistically significant ($p<0.0001$)

Not only was time to morbidity significantly enhanced by A6 treatment, lymph node involvement of tumor was significantly reduced by up to 70% (P=0.004-See Boyd et al, Fig. 5-APPENDIX. These results demonstrate inhibition of uPAR has therapeutic potential for the treatment of prostate cancer.

Inhibition of c-Met

We also demonstrated that inhibition of c-Met led to inhibition of uPaR expression. Thus, one of the other parts of Task 2 was to determine the effect of c-Met inhibition on growth and metastasis of prostate cells in the identical orthotopic model. Prior to performing "gene therapy" experiments, to examine the feasibility of c-Met inhibition, PC3-LN 4 cells were infected ex vivo with a control or ribozyme-expressing virus, and implanted orthotopically in nude mice. Results from this set of experiments are shown in Table 1 (Kim et al., 2003-Table 1-see Appendix).

Table 1 *Tumorigenicity and lymph node metastases induced by PC3-LN4 Cells following ex vivo Infection of Adenovirus or Mock Infection*

Group[a]	Tumor Incidence[b]	Tumor size (g) median (range)	Lymph node Involvement[c]
Mock	8/8	0.58 (0.31-1.01)	8/8
Ad-Pu-1	9/9	0.47 (0.01-2.45)	9/9
Ad-c-Met	1/10	0.07	0/1

[a]PC 3-LN4 cells were mock infected, or infected with the indicated adenovirus at an MOI of 50, and 5 x 10^5 cells were injected into the prostates of nude mice, as described in Materials and Methods

[b]Number of mice with tumors/number of mice surviving surgery for prostate tumor cell implantation

[c]Number of mice with lymph node metastases/number of mice with tumors

These results were published as a feature paper in Clinical Cancer Research, accompanied by a commentary (Shinomiya N and Vande Woude GF, 2003) and demonstrate the importance of c-Met as a therapeutic target for prostate cancer progression.

Inhibition of c-Met-mediated Signaling by PTEN/MMAC

UPaR is one of the downstream targets of c-Met signaling. During the course of the work, we also explored inhibition of c-Met signaling through use of re-expression of the tumor suppressor gene PTEN/MMAC. As the ultimate goal of this project was to inhibit tumor spread, and non-DOD sources of funding were also available for this study, we pursued these studies in the context of our c-Met work. Results from our study were striking, with ectopic expression of c-Met inhibiting tumor metastasis, but not tumor establishment of PC3 cells in an orthotopic nude mouse model (Davies et al., 2002). Thus, it is possible that re-introduction of MMAC/PTEN will be an excellent combination strategy to those that are the main focus of the Statement of Work, and the significance of this work was discussed in a commentary on our paper by Fernandez and Eng (2002).

Task 3

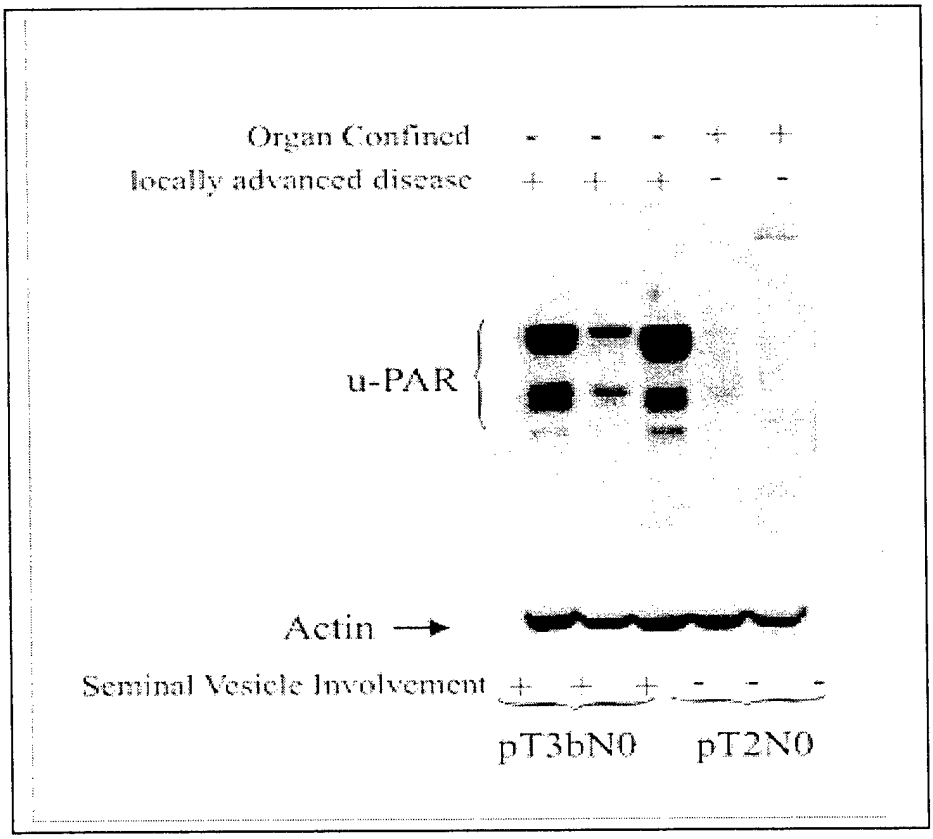

Fig. 2 Expression of uPaR in human prostate tumor tissue. Tissue lysates were made and uPaR expression was determined by Western blotting.

This task involved examination of surgical specimens. While we hope to acquire more specimens for this task, we have achieved important results with respect to uPAR expression in stages of prostate cancer. These are presented in Figure 2. As can be seen in the Figure, uPaR expression is considerably elevated in locally advanced disease versus organ-confined disease. These results confirm that uPaR overexpression correlates with progressed stages of

CaP. We are currently examining uPa and c-Met expression in these samples, but others have shown that c-Met expression also correlates with progressive stages of disease.

KEY RESEARCH ACCOMPLISHMENTS

- Reduction of c-Met decreases uPAR expression
- Inhibition of uPAR function alone is sufficient to inhibit migration and invasion of human prostate tumor cells
- Inhibition of c-Met expression alone is sufficient to inhibit migration and invasion of human prostate tumor cells
- Reduction of c-Met expression decreases tumorigenicity and metastatic potential of human prostate cells in an orthotopic model
- Gene therapy experiments in nude mice demonstrate that c-Met reduction not only inhibits growth of prostate tumors, but blocks development of lymph node metastasis
- Ectopic expression of PTEN/MMAC, a downstream regulator of c-Met signaling, inhibits growth and development of metastases of human prostate tumor cells in an orthotopic model
- Treatment of mice with the uPAR antagonist A6 significantly increases survival (as measured by onset of morbidity) of nude mice harboring tumors induced by human prostate tumor cells
- uPaR expression increases with invasive stages of CaP in human tumor tissue.

REPORTABLE OUTCOMES

The hypotheses posed in the original submission with respect to c-Met regulating uPAR and promoting invasion and metastasis of prostate cancer appear correct. In addition, as described last year, we have begun to define the relationship between c-Met, uPAR, and the metastasis suppressor, MMAC/PTEN. These data are now in publication in three manuscripts, two of which were feature papers with commentaries:

Adenoviral-mediated expression of MMAC/PTEN inhibits proliferation and metastasis of human prostate cancer cells. Davies, MA, Kim SJ, Parikh NU, Dong, Z., Bucana CD, and **Gallick, GE** ***Clinical Cancer Res***, **8**, 1904-1914, 2002.

A Urokinase-Derived Peptide (A6) Increases Survival of Mice Bearing Orthotopically Grown Prostate Cancer and Reduces Lymph Node Metastasis. Boyd, D.D., Kim, S.-J., Wang, H., Jones, T.R., and **Gallick, G.E. Am. J. Pathol**. 162, 619-626, 2003.

Reduced c-Met Expression by an Adenovirus Expressing a c-Met Ribozyme Inhibits Tumorigenic Growth and Lymph Node Metastases of PC3-LN4 Prostate tumor Cells in an Orthotopic Nude Mouse Model. Kim S.-J., Johnson, M., Koterba K., Uehara H., and **Gallick, G.E.** ***Clinical Cancer Research***, 9,5161-5170, 2003.

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CONCLUSIONS

Both uPAR and c-Met contribute to the invasive potential of prostate tumor cells; c-Met also contributes to the migratory potential of these cells. Both c-Met and uPAR are targets for therapeutic strategies to inhibit prostate cancer. In addition, "downstream" signaling pathways regulated by c-Met may also be targets for the development of inhibitors. Inhibition of c-Met results in inhibition of growth at the primary tumor site (prostate), and significantly, prevents development of lymph node metastases in an orthotopic nude mouse model. Several strategies are currently being developed to inhibit uPaR expression and c-Met expression and/or activity, including the development of small molecule inhibitors in various stages of preclinical trial. The results from the funded studies of DAMD17-00-1-0524 suggest strongly that these inhibitors may have clinical efficacy in prostate cancer.

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Kim S.-J., Johnson, M., Koterba K., Uehara H., and **Gallick, G.E**. Reduced c-Met Expression by an Adenovirus Expressing a c-Met Ribozyme Inhibits Tumorigenic Growth and Lymph Node Metastases of PC3-LN4 Prostate tumor Cells in an Orthotopic Nude Mouse Model. ***Clinical Cancer Research***, 9:5161-5170, 2003.

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Adenoviral-mediated Expression of MMAC/PTEN Inhibits Proliferation and Metastasis of Human Prostate Cancer Cells[1]

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ABSTRACT

Purpose: **The purpose of this study was to determine the effects of adenoviral transgene expression of MMAC/PTEN on the *in vitro* and *in vivo* growth and survival of PC3 human prostate cancer cells.**

Experimental Design: **Adenoviruses expressing MMAC/PTEN or green fluorescent protein as a control were introduced into PC3 cells, and effects on signal transduction pathways and growth of tumors in an orthotopic nude mouse model were determined.**

Results: **MMAC/PTEN expression in PC3 cells decreased the level of phospho Akt but not that of phospho Mapk or FAK. Expression of MMAC/PTEN inhibited the *in vitro* growth of PC3 cells primarily by blocking cell cycle progression. *Ex vivo* introduction of MMAC/PTEN expression did not inhibit the tumorigenicity of orthotopically implanted PC3 cells, but it did significantly reduce tumor size and completely inhibited the formation of metastases. *In vivo* treatment of pre-established orthotopic PC3 tumors with adenoviral MMAC/PTEN did not significantly reduce local tumor size, but it did diminish metastasis formation.**

Conclusions: **MMAC/PTEN functionally regulates prostate cancer cell metastatic potential in an *in vivo* model system and may be an important biological marker and therapeutic target for human prostate cancer.**

INTRODUCTION

Prostate cancer is the second leading cause of cancer-related deaths in men in the United States, with a predicted 32,000 patients succumbing to the disease in 2001 (1). Currently, there is no effective treatment for advanced stages of this disease. To improve our understanding of prostate cancer progression, many studies have examined the chromosomal alterations that characterize its different stages (2, 3). Although many alterations have been observed, those involved most frequently include chromosomes 7q, 8p, 10, 13q, and 16q (4–6). Allelic deletions associated with chromosome 10q, specifically the q23–25 region, have been observed to preferentially occur in the advanced stages of disease (7, 8). Reintroduction of this chromosomal region into rat prostate cancer cells significantly inhibited their metastatic capabilities but failed to alter their tumorigenicity (9). Many studies have implicated the tumor suppressor gene, *MMAC/PTEN,* as critical to the biological effects associated with deletions in (or reintroduction of) chromosome 10. *MMAC/PTEN* was initially identified because of homozygous deletions of the gene in human glioma and breast cancer cell lines, as well as by its homology to protein phosphatases (10–12). These and subsequent studies demonstrated that deletions and mutations of the gene occur in a wide variety of cancers, including glioma, prostate, breast, melanoma, endometrial, and ovarian (reviewed in Ref. 13). With the exception of endometrial cancer, alterations to MMAC/PTEN are detected almost exclusively in advanced stages of disease. Thus, the loss of *MMAC/PTEN* function does not appear to be required for tumor initiation in these diseases but instead was a hallmark of tumor progression.

One of the critical functions for the tumor suppressor activity of MMAC/PTEN is its intrinsic lipid phosphatase activity (14), which results in removal of the phosphate moiety at the 3′ position of phosphatidylinositol. By this activity, MMAC/PTEN antagonizes signaling mediated by PI3K[3] and negatively regulates the phosphorylation and activation of a number of important mitogenic and/or prosurvival signaling molecules. Our group and others have shown that enforced MMAC/PTEN expression decreases the phosphorylation and kinase activity of Akt/PKB, a proto-oncogene that is activated by PI3K-mediated signaling events (15, 16). Akt/PKB, a serine-threonine protein kinase, has been shown to be a critical regulator of many different cellular processes, including apoptosis, cell cycle progression, angiogenesis, and metabolism. Other reports (17, 18) have demonstrated that MMAC/PTEN may also dephosphorylate FAK, a protein tyrosine kinase involved in the regulation of cellular adhesion, motility, and survival.

Numerous studies have implicated the loss of MMAC/

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[3] The abbreviations used are: PI3K, phosphatidylinositol-3′ kinase; MAPK, mitogen-activated protein kinase; Ad-GFP, adenovirus expressing the enhanced green fluorescent protein; Ab, antibody; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; MOI, multiplicity of infection; FAK, focal adhesion kinase; FACS, fluorescence-activated cell sorter; PKB, protein kinase B.

PTEN in prostate cancer progression. In the initial identification of the gene, homozygous genetic inactivation of MMAC/PTEN was observed in the LNCaP and PC3 human prostate cancer cell lines, both of which were established from metastatic lesions (10, 11). Multiple cytogenetic studies of prostate tumors detected low rates of mutation and deletion of MMAC/PTEN in organ-confined prostate cancers (19–21). However, ~60% of metastatic prostate cancers demonstrated loss of heterozygosity for *MMAC/PTEN,* accompanied by a significant rate of alterations of the second allele (22, 23). In several tumors that retain the *MMAC/PTEN* gene, decreased expression of the protein may be observed, *e.g.,* Whang *et al.* (24) demonstrated that *MMAC/PTEN* mRNA and protein levels were down-regulated in >50% of prostate tumors in which the *MMAC/PTEN* coding region was not mutated. Furthermore, immunohistochemical analysis of human prostate cancer specimens detected down-regulated expression of MMAC/PTEN protein in a heterogeneous pattern (25). Interestingly, the foci in which MMAC/PTEN expression was lost were generally high-grade lesions, suggesting that these foci might give rise to the aggressive components of these tumors.

In contrast, studies using genetically engineered mice suggest a potential role for MMAC/PTEN at an earlier stage of prostate tumor development. The loss of both alleles of *MMAC/PTEN* results in embryonic lethality in mice (26–28). *MMAC/PTEN*$^{+/-}$ mice are viable and spontaneously develop lymphoid hyperplasia, several types of tumors, and an autoimmune syndrome (29). The prostates of these mice demonstrate global hyperplasia and frequently develop prostatic intraepithelial hyperplasia, the precursor of invasive prostate cancer. The global dysregulated growth suggests that the loss of a single allele of *MMAC/PTEN* causes this phenotype (26). Breeding these mice with *p27*$^{+/-}$ mice, leading to progeny that are genetically *MMAC/PTEN*$^{+/-}$ and *p27*$^{+/-}$ or $^{-/-}$, resulted in 100% penetrance of frank prostate cancer, including invasive tumors (30). Thus, these knockout studies suggest that *MMAC/PTEN* is a potent regulator of prostate proliferation and demonstrate in this system a potential role in the earlier stages of prostate cancer tumorigenesis.

To further examine the ability of MMAC/PTEN expression to regulate prostate tumor development and/or progression, we have studied the effects of MMAC/PTEN expression in PC3 human prostate cells. The PC3 cell line was originally derived from a prostate cancer metastasis and represents a model for androgen-insensitive disease. The PC3 cells lack MMAC/PTEN expression because of deletion of both alleles (10, 11) and also lack wild-type p53 function and androgen receptor expression (31). When injected orthotopically into the prostate of nude mice, the PC3 cells grow aggressively locally (32, 33) and readily metastasize to regional lymph nodes (31). In this study, we expressed MMAC/PTEN in PC3 cells via an adenovirus vector Ad-MMAC (15) and examined the effects on cell signaling pathways, growth, and survival of PC3 cells *in vitro*. Additionally, the effects of MMAC/PTEN expression on the *in vivo* growth of PC3 cells was tested both by orthotopic implantation of cells infected with Ad-MMAC before their inoculation into nude mice (*ex vivo* treatment) and by the treatment of preestablished tumors with Ad-MMAC (*in vivo* treatment). These studies provide further insight into the ability of MMAC/PTEN to regulate specific cell signaling pathways and cellular behaviors in prostate cancer cells and demonstrate for the first time the regulation of *in vivo* prostate cancer metastatic capability.

MATERIALS AND METHODS

Cell Lines and Adenoviruses. The PC3 cell line was obtained originally from the American Type Culture Collection, Rockville, MD. The cells were maintained in culture in media supplemented with 10% FCS and incubated in 5% CO_2/95% air at 37°C. The recombinant adenovirus Ad-MMAC expresses the human wild-type MMAC cDNA under the control of the human cytomegalovirus immediate-early promoter/enhancer (34) and was generously provided by Robert Bookstein (Canjii, Inc., San Diego, CA). Ad-GFP was derived from the same vector as Ad-MMAC; the replication-deficient virus without a transgene (Ad-DE1/Ad5-δE1) has been described previously (35), along with the virus expressing the p53 transgene (36). Adenoviruses were harvested after infection of 293 cells and isolated by cesium chloride gradient, and titer was determined by absorbance. Briefly, viral stock was propagated in 293 cells. Cells were harvested 36–40 h after infection, pelleted, resuspended in PBS, and lysed by three freeze-thaw cycles. Cell debris was removed, and resulting cellular lysates were subjected to double CsCl centrifugation. Concentrated virus was dialyzed, aliquoted, and stored at −80°C. Adenoviral titer represented as optical particles units was determined by optical absorbance as described previously (37).

Tumorigenicity Studies. Cells (500,000) were plated on 10-cm tissue culture dishes. Approximately 18 h later, cells were infected with indicated amounts of adenovirus in 4 ml of media. Cells were gently agitated every 15 min for 1 h, then 6 ml of fresh media were added to each plate. Cells were infected with Ad-MMAC, or Ad-GFP at the indicated MOI, or mock infected by incubation with media alone. Twenty-four h after infection, cells were harvested by trypsinization. Cells were counted and resuspended in Ca^{2+} and Mg^{2+}-free HBSS at 100,000 cells/50 μl. Cells were then implanted in the prostate of nude mice as described previously (33). In brief, nude mice were anesthetized with Nembutal and placed in a supine position. A low-midline incision was made, and the prostate was exposed. Fifty μl of HBSS containing 100,000 cells were injected into a lateral lobe of the prostate. The wound was closed with surgical metal clips. Each experimental condition was performed on groups of eight mice.

Gene Therapy Studies. Subconfluent cultures of PC3 cells were harvested by trypsinization. Cells were counted, then were resuspended in Ca^{2+} and Mg^{2+}-free HBSS at 50,000 cells/50 μl. Cells were then implanted in the prostate of nude mice as described above, and the wound was closed with surgical metal clips. Tumors were treated at 7 and 14 days after implantation. Tumors were exposed by the same procedure, by a low-midline incision, and exposure of the prostate. Tumors were injected with PBS, or 1.5×10^9 plaque-forming units of Ad-GFP or Ad-MMAC, each in a total volume of 20 μl. Tumors and lymph nodes were harvested 14 days after the final gene intratumoral injection. Each experimental condition was performed on groups of eight mice.

Necropsy Procedures and Histological Studies. The mice were euthanized at the times indicated above. Primary tumors in the prostate were excised and weighed. For immunohistochemistry and H&E staining procedures, some tumors were embedded in OCT compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70°C. Other tumors were formalin fixed and paraffin embedded. Lymph nodes were harvested, and the presence of metastatic disease was determined histologically.

For BrdUrd staining, the mice were injected with 250 μg of BrdUrd. Two h later, the mice were sacrificed, and the prostates were formalin fixed and embedded in paraffin. For staining, tissue sections were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were then treated with 2N HCl for 30 min at 37°C. For CD31 staining, sections of frozen tissues were fixed with cold acetone and transferred to PBS. The slides were then rinsed twice with PBS, and endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 12 min. The samples were then washed three times with PBS and incubated for 10 min at room temperature with a protein-blocking solution consisting of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum. Excess blocking solution was drained, and the samples were incubated for 18 h at 4°C with 1:100 dilution of monoclonal mouse anti-BrdUrd Ab (Becton Dickinson, Mountain View, CA) or a 1:100 dilution of monoclonal rat anti-CD31 Ab (PharMingen, San Diego, CA). The samples were then rinsed four times with PBS and incubated for 60 min at room temperature with the appropriate dilution of peroxidase-conjugated antimouse IgG1 or antirat IgG. The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics, Huntsville, AL). The sections were then washed three times with distilled water and counterstained with Gill's hematoxylin.

For TUNEL staining, frozen sections were fixed with 4% paraformaldehyde in PBS for 20 min, rinsed twice with PBS, and rinsed with double distilled water containing 0.04% Brij-35 (Fisher Scientific, Pittsburgh, PA). The sections were treated with 20 μg/ml Proteinase K (Boehringer Mannheim) for 10 min, rinsed twice with PBS for 5 min, and incubated with terminal deoxynucleotidyl transferase buffer for 10 min. Terminal deoxynucleotidyl transferase and biotinylated 16-dUTP (Boehringer Mannheim) were then added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 h. The slides were washed with buffer two times in 5 min, followed by double distilled water. The sections were incubated with a protein block consisting of 2% normal goat serum in PBS for 10 min, followed by peroxidase-labeled streptavidin diluted 1:400 for 30 min at 37°C. The slides were rinsed three times with PBS and incubated with the Stable 3,3′-diaminobenzidine (Research Genetics) for 30 min at 37°C. The sections were rinsed with distilled water, air dried, mounted with Universal Mount Research Genetics), and examined by bright field microscopy.

Cell Growth. Cells (500,000) were plated on 10-cm tissue culture dishes. Approximately 18 h later, cells were infected with indicated amounts of adenovirus in 4 ml of media. Cells were gently agitated every 15 min for 1 h, then 6 ml of fresh media were added to each plate. Cells were infected with Ad-GFP, Ad-MMAC, or Ad-p53 at the indicated MOI or mock infected with media alone. At the indicated time points, cells were harvested by collecting the supernatant and trypsinizing attached cells. Viable cells were counted by microscopic analysis using a hemocytometer. The significance of differences in cell number was analyzed by unpaired Student's *t* test.

Protein Analysis. Cells (500,000) were plated on 10-cm tissue culture dishes. Approximately 18 h later, cells were infected with indicated amounts of adenovirus in 4 ml of media. Cells were gently agitated every 15 min for 1 h, then 6 ml of fresh media were added to each plate. Cells were infected with Ad-GFP or Ad-MMAC at the indicated MOI or mock infected by incubation with media alone. At the indicated time points, cells were harvested on ice. For analysis of signal transduction pathways, 24 h after infection, cells were washed with serum-free media twice, then incubated overnight in serum-free media. After ~18 h of serum starvation, cells were harvested either with ("stimulated") or without ("starved") a 10-min incubation with 1% FCS-enriched media. Harvesting of cell lysates was performed on ice. After two washes with cold 1× PBS, cells were harvested in lysis solution containing 50 mM HEPES (pH 7.0), 150 mM NaCl, 1 mM EGTA, 100 mM NaF, 10 mM $NaPP_i$, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 μM pepstatin, 10 μg/ml aprotinin, 5 mM iodoacetic acid, and 2 μg/ml leupeptin. The homogenates were clarified by centrifugation at 15,000 × *g* for 15 min at 4°C.

Western analysis was performed as described previously (16). Immunoblotting was performed using antibodies against total and phospho-specific Akt and MAPK (New England Biolabs, Boston, MA) and MMAC/PTEN (Santa Cruz Biotechnology, Santa Cruz, CA), followed by horseradish peroxidase-conjugated secondary Ab (Amersham, Chicago, IL). Protein-Ab complexes were detected by chemiluminescence (enhanced chemiluminescence; Amersham). For detection of MMAC/PTEN in mouse tumors, 500 μg of tumor lysate were first immunoprecipitated with the MMAC/PTEN Ab (Santa Cruz Biotechnology); then Western blotting was performed as described above.

For analysis of FAK phosphorylation, 250 μg of lysate were incubated with 2 μg of α-FAK monoclonal Ab (Upstate Biotechnology) for 2.5 h at 4°C, with 6 μl of rabbit antimouse IgG Ab for 1 h, then with 35 μl of Pansorbin Cells for 30 min. Complexes were centrifuged for 1 min at 15,000 × *g*, washed three times with immune complex kinase assay wash buffer, boiled for 5 min in 1× SDS-PAGE buffer, then run on 7.5% gels and transferred to Immobilon-P membranes as described above. Immunoblotting was performed using antibodies specific for phospo-tyrosine residues (4G10; Upstate Biotechnology) and FAK (Transduction Labs).

Apoptosis and Cell Cycle Analysis. Cells (500,000) were plated on 10-cm tissue culture dishes. Approximately 18 h later, cells were infected with indicated amounts of adenovirus in 4 ml of media. Cells were gently agitated every 15 min for 1 h; then 6 ml of fresh media were added to each plate. Cells were infected with Ad-GFP, Ad-DE1, Ad-MMAC, or Ad-p53 at the indicated MOI or mock infected with media alone. At the indicated time points, cells were harvested by collecting the supernatant and trypsinizing attached cells. Cells were fixed in 1% paraformaldehyde and stored in 70% ethanol at 4°C. An equal number of cells was then processed and analyzed for

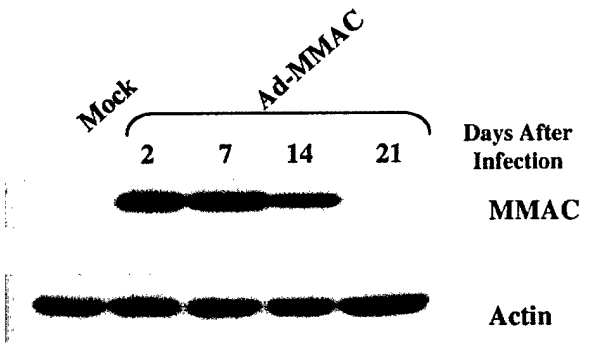

Fig. 1 MMAC/PTEN expression in PC3 cells. Western blotting analysis of PC3 treated with media alone (*Lane 1*) or infected with 25 MOI Ad-MMAC (*Lanes 2–5*). Ad-MMAC-infected cells were harvested at 2, 7, 14, and 21 days after infection to determine induction and duration of MMAC/PTEN expression (*top*). Immunoblotting for actin (*bottom*) performed to confirm protein loading.

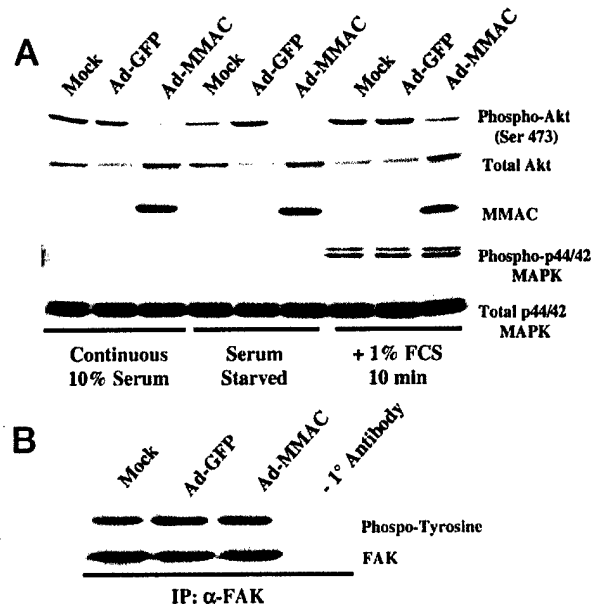

Fig. 2 Effects of ectopic MMAC/PTEN expression in PC3 cells on expression and phosphorylation of signaling intermediates. In *A*, immunoblotting was performed 48 h after mock infection or infection with 25 MOI of Ad-GFP or Ad-MMAC. Cells were harvested after continuous incubation in complete tissue culture media (*Lanes 1–3*) or after a 24-h serum starvation with (*Lanes 7–9*) or without (*Lanes 4–6*) acute 1% FCS stimulation. Phosphorylation of Akt/PKB and p44/42 MAPK (*bottom*) was detected by phosphorylation site-specific antibodies. MMAC/PTEN protein expression also shown (*middle*). *B*, phosphorylation status of FAK in PC3 cells mock treated with media alone (*Lane 1*) or infected with 25 MOI of Ad-GFP (*Lane 2*) or Ad-MMAC (*Lane 3*). Cells were harvested 48 h after indicated treatments. Cell lysates were immunoprecipitated with α-FAK, immunoblotted with α-phosphotyrosine (*top*), and reprobed with α-FAK (*bottom*).

apoptosis by TUNEL assay using the Apo-BrdUrd kit (Phoenix Flow Systems, San Diego, CA) or stained solely with propidium iodide (50 μg/ml in PBS). Apoptotic cells were detected and quantitated by flow cytometry. The significance of differences between different treatment groups was analyzed by unpaired Student's *t* test.

RESULTS

***In Vitro* Studies.** PC3 cells were chosen for these studies because of their ability to form lymph node metastases after orthotopic implantation into nude mice (33). Before the animal studies, we first examined whether the effects of ectopic expression of PTEN/MMAC were similar to those observed in other cell lines. Flow cytometric analysis of PC3 cells infected with an adenovirus engineered to express the Ad-GFP determined that 25 MOI was the minimum concentration of adenovirus required to infect >95% of the cells. To express MMAC/PTEN, PC3 cells were infected with a replication-deficient adenovirus engineered to express wild-type *MMAC/PTEN* cDNA under the control of the human cytomegalovirus promoter. Western blotting analysis showed that PC3 cells did not express MMAC/PTEN protein after mock treatment or infection with a control adenovirus but express high levels after infection with Ad-MMAC (Fig. 1). MMAC/PTEN expression was maintained for at least 21 days in cultured cells, although a significant decrease in the amount of protein was observed at later time points, as compared with 48 h after infection. It is not clear if this represents a decrease in expression by cells infected with Ad-MMAC or a dilutional effect of the proliferation of cells that were not infected.

To further characterize the function of MMAC/PTEN in PC3 cells, the effects of its expression on the phosphorylation status of intermediates of cell signaling pathways were examined by Western blotting. Analysis was performed on cells maintained in FCS-enriched media, as well as in cells that were serum starved or acutely stimulated. For all three tissue culture conditions, MMAC/PTEN expression inhibited phosphorylation of Akt/PKB at its activating residues without diminishing total Akt/PKB protein levels (Fig. 2*A*). These results are consistent with MMAC/PTEN's lipid phosphatase activity and are identical to our previous results in both LNCaP prostate cancer cells and human glioma cells (15, 16). The Ad-GFP virus also led to small reductions in phospho Akt expression; however, this reduction was substantially less than that observed with the Ad-MMAC virus. MMAC/PTEN expression did not decrease the mitogen-stimulated phosphorylation of p44/42 MAPK, as compared with mock or control adenovirus treatments. This also is consistent with our previous results. The effect of MMAC/PTEN expression on the phosphorylation of FAK was also examined. No decrease was observed in tyrosine phosphorylation, or total protein levels, of FAK in PC3 cells (Fig. 2*B*).

The ability of MMAC/PTEN to regulate PC3 cellular growth *in vitro* was determined by counting viable cell numbers at various time points after infection with control adenoviruses or Ad-MMAC or mock treatment with media alone. Infection with Ad-MMAC decreased the rate of PC3 proliferation at all time points examined (Fig. 3*A*). Treatment with Ad-MMAC was significantly more growth inhibitory than treatment with a control adenovirus (Fig. 3*B*; $P < 0.05$ on day 4, $P < 0.01$ on day 6). In previous studies, we had observed that MMAC/PTEN expression was more growth inhibitory than p53 expression in

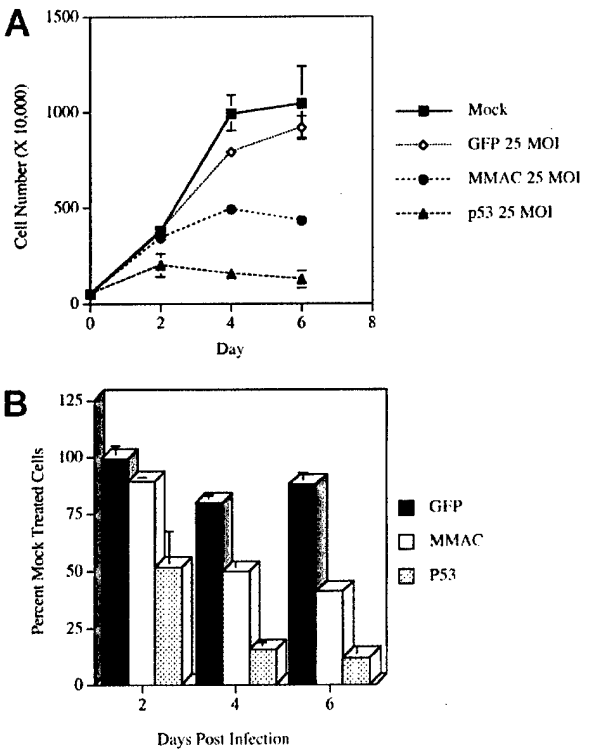

Fig. 3 Effects of ectopic MMAC/PTEN and p53 expression on *in vitro* PC3 cell growth. *A*, viable number of PC3 cells mock infected (■) or infected with 25 MOI of Ad-GFP (◇), Ad-MMAC (●), or Ad-p53 (▲) at 0, 2, 4, and 6 days after infection. *B*, growth of PC3 cells infected with 25 MOI of Ad-GFP (*solid bars*), Ad-MMAC (*open bars*), or Ad-p53 (*shaded bars*), as compared with mock-infected cells. *Columns*, average; *bars*, SD.

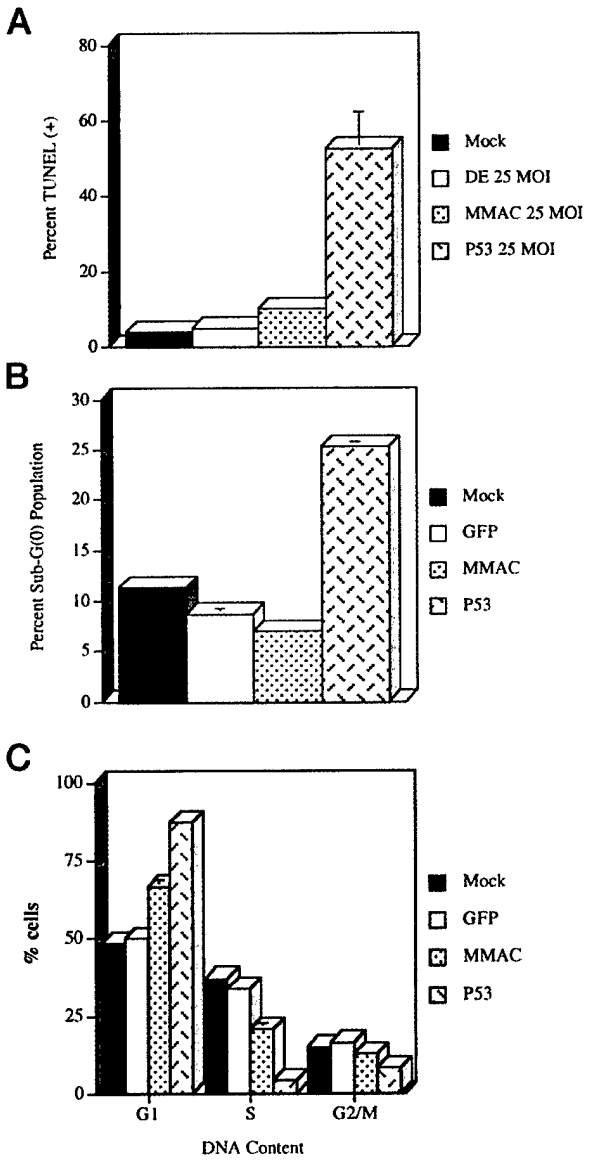

Fig. 4 Effects of ectopic MMAC/PTEN and p53 expression on *in vitro* PC3 apoptosis. In *A*, graph shows the percentage of PC3 cells that stain TUNEL positive after mock infection (*solid bars*) or infection with 25 MOI of Ad-GFP (*open*), Ad-MMAC (*shaded*), or Ad-p53 (----) at day-4 postinfection. In *B*, graph shows the percentage of PC3 cells with Sub-G_0 DNA content at day 6 after infection, as determined by propidium iodide staining followed by FACS analysis. Identical labels as in *A*. *Columns*, average; *bars*, SD. *C*, cell cycle distribution of PC3 cells harvested 58 h after infection, as determined by phosphatidylinositol staining followed by FACS analysis. Cells were mock infected (*solid bars*) or infected with 25 MOI of Ad-GFP (*open*), Ad-MMAC (*shaded*), or Ad-p53 (----). *Columns*, average; *bars*, SD.

LNCaP prostate cancer cells (16). Therefore, the effect of Ad-MMAC treatment on the growth of PC3 cells was compared with treatment with adenoviral p53 (Ad-p53). In contrast to LNCaP cells, PC3 cells were more sensitive to p53 expression than to ectopic MMAC/PTEN expression. Infection with Ad-p53 resulted in an 85% reduction in viable cell number at day 4 and ~90% at day 6 after infection. Both of these effects were significantly greater than was seen with Ad-MMAC ($P < 0.05$ for both days).

To determine the mechanism of MMAC/PTEN-mediated growth inhibition, the effects of these treatments on apoptosis and proliferation were examined by flow cytometry (Fig. 4). Four days after infection with Ad-MMAC, 10% of cells were TUNEL positive, as compared with 5% with control adenovirus, suggesting only a minor increase in MMAC/PTEN-induced apoptosis. In contrast, infection with Ad-p53 produced 52% TUNEL positivity, indicating a significant induction of apoptosis by p53 expression (Fig. 4*A*). As an independent measure of apoptosis, propidium iodide staining followed by FACS analysis was performed to determine the percentage of cells with sub-G_0 DNA content (*i.e.*, cells likely to be undergoing apoptosis). On day 6 after infection, only 7% of Ad-MMAC-infected cells had sub-G_0 DNA content, as opposed to 25% of Ad-p53-treated cells (Fig. 4*B*). Similar differences in relative apoptosis were observed in both assays, suggesting that the growth-inhibitory effect induced by MMAC/PTEN expression cannot be attributed to the induction of apoptosis.

The effect of MMAC/PTEN expression on cellular proliferation was examined by cell cycle analysis (Fig. 4*C*). Infection

with Ad-MMAC produced an increase in the G_1 population of ~20%, with a concurrent decrease in the S phase population *versus* mock-treated cells. The changes in both the G_1 and S phase populations after MMAC/PTEN expression were significant when compared with either mock treatment or infection with a control adenovirus ($P < 0.05$). This G_1 arrest is consistent with experiments examining MMAC/PTEN expression in other cell types (38–42). p53 expression also induced a G_1 arrest in the PC3 cells. Similar to the assays of cell growth and apoptosis, the arrest induced by p53 expression was significantly greater than that induced by MMAC/PTEN, as quantified by G_1 and S phase cell populations ($P < 0.05$).

***In Vivo* Studies.** Previous studies in a number of human cancer cell types have demonstrated that the orthotopic implantation of cells in nude mice more closely resembles the biological behaviors of these cells in humans, particularly in regards to the development of metastases. This has proven particularly true of human prostate cancer cells, which form primary tumors and metastases with much lower efficiency when implanted ectopically in nude mice.

To assess the ability of MMAC/PTEN expression to regulate PC3 tumorigenicity and *in vivo* growth, the effects of *ex vivo* treatment of the cells were examined. PC3 cells were mock infected, or infected with 25 MOI of Ad-GFP or Ad-MMAC, as had been performed for the *in vitro* studies. After (24 h) these treatments, equal numbers of cells were injected into the prostates of nude male mice. The mice were then sacrificed 21 days after implantation, as was necessitated by the morbidity resulting from the tumors that had formed. The mice were dissected and analyzed for the incidence and size of prostate tumors and for the incidence of metastases to regional lymph nodes. A 100% incidence of prostate tumors was observed for all three treatments, indicating that MMAC/PTEN expression did not inhibit PC3 tumorigenicity. Although MMAC/PTEN expression did not abolish tumor formation, the size of tumors resulting from Ad-MMAC-infected cells was significantly smaller than tumors of the other treatment groups (Fig. 5*A*). Tumors formed by PC3 cells infected with Ad-MMAC were 77% smaller by mass than tumors formed from mock-infected PC3 cells, whereas Ad-GFP-infected cells were only 25% smaller. In addition to its effect on local tumor growth, MMAC/PTEN expression completely inhibited the development of PC3 lymph node metastases. Although 100% of the mice in the mock treatment group and 80% of the mice in the Ad-GFP treatment group demonstrated enlarged lymph nodes, with the presence of PC3 cells confirmed histologically, none of the mice in the Ad-MMAC treatment group demonstrated lymph node metastases (Fig. 5*B*). Thus, MMAC/PTEN expression did not inhibit primary tumor formation, but it did reduce primary tumor size and inhibited the development of lymph node metastases.

Immunohistochemical analysis was performed on the harvested tissues to determine the effects of MMAC/PTEN expression on the *in vivo* behavior of PC3 cells. MMAC/PTEN expression did not affect the morphology of the PC3 cells, or the general architecture of the tumors formed by these cells, as demonstrated by H&E staining (Fig. 6*A*). These sections also demonstrate the histological evidence of lymph node invasion by mock- and Ad-GFP-infected PC3 cells, whereas the lymph nodes of Ad-MMAC-infected cells demonstrate only lymphoid

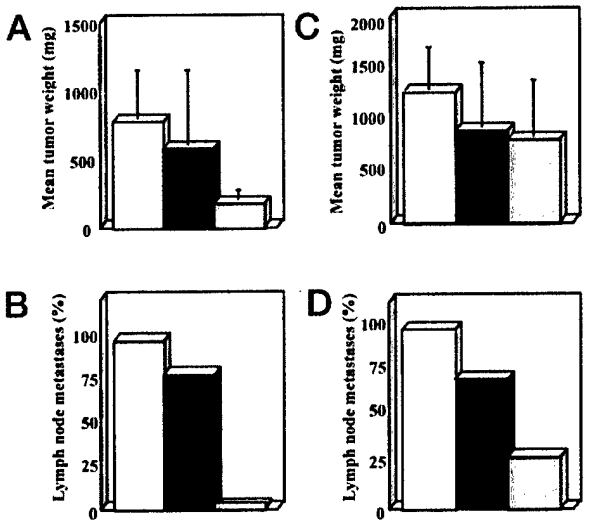

Fig. 5 Effects of MMAC/PTEN expression on the growth and metastasis of orthotopically implanted PC3 cells. For *ex vivo* studies, 24 h before implantation, PC3 cells were mock infected with media alone (*open bar*) or were infected with 25 MOI of Ad-GFP (*solid bar*) or Ad-MMAC (*shaded bar*). Tumors were harvested 21 days after implantation. *A*, mean tumor weight; *B*, percentage of mice with histologically confirmed lymph node metastases. *Columns*, average; *bars*, SD. For *in vivo* Ad-MMAC treatment of pre-established orthotopic PC3 tumors on growth and metastasis, tumors were injected with indicated treatments at days 7 and 14 after implantation and were harvested 17 days after the final treatment. Graphs demonstrate mean tumor weight (*C*) and percentage of mice with lymph node metastases (*D*) of PC3 tumors treated *in vivo* with PBS (*open bar*), Ad-GFP (*solid bar*), or Ad-MMAC (*shaded bar*). *Columns*, average; *bars*, SD.

cells (Fig. 6*B*). To determine the mechanism of MMAC/PTEN's inhibitory effect on primary tumor size, the tumors were stained for markers of apoptosis, angiogenesis, and proliferation. TUNEL staining for apoptotic cells did not demonstrate differences between the three treatment groups (Fig. 7*A*). To assess tumor angiogenesis, the tumors were stained for CD31, a marker of endothelial cells. Again, no significant differences were observed between the different treatments (Fig. 7*B*). Finally, tumor proliferation was assessed by BrdUrd incorporation. Tumors of both mock- and Ad-GFP-treated PC3 cells showed generalized, strongly positive staining for BrdUrd, indicating that the tumor cells were proliferating globally (Fig. 7*C*). In contrast, most of the areas of the Ad-MMAC treatment group tumors were negative for BrdUrd incorporation, although there were some positively stained regions around the tumor margin. Thus, *ex vivo* MMAC/PTEN expression significantly inhibited PC3 cellular proliferation *in vivo*, without significantly affecting apoptosis or angiogenesis. Western blotting analysis of immunoprecipitated proteins indicated that the tumors of PC3 infected with Ad-MMAC *ex vivo* did express elevated levels of MMAC/PTEN at the time of tumor harvest (data not shown).

As *ex vivo* treatment of PC3 cells with Ad-MMAC reduced primary tumor size and inhibited metastasis formation, we next tested the effects of *in vivo* Ad-MMAC treatment of pre-established PC3 orthotopic tumors. PC3 cells were injected into the prostate of nude mice. The tumors were injected on days 7 and

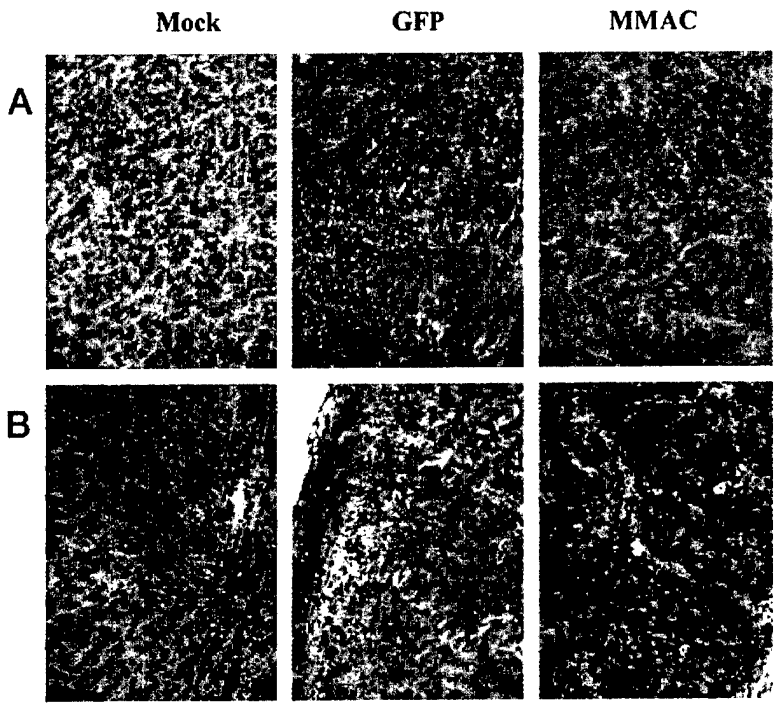

Fig. 6 Effect of *ex vivo* MMAC/PTEN expression on morphology of orthotopic PC3 tumors and histological examination of regional lymph nodes. H&E staining of frozen prostate tumors (*A*) and regional lymph nodes (*B*) harvested 21 days after implantation. Prostates for all treatment groups demonstrate replacement of normal tissue by tumor cells. Lymph nodes of mock- and Ad-GFP-infected PC3 cells demonstrate evidence of metastasis, as most of the tissue consists of prostate cancer cells, with few normal lymphoid cells (small, *blue* cells) remaining. The lymph node of the Ad-MMAC treatment group demonstrates no evidence of metastasis.

14 after implantation with 1.5×10^9 plaque-forming units of Ad-GFP or Ad-MMAC or an equal volume of PBS. Mice were sacrificed at day 31 after implantation, because of the morbidity of the tumor burden. None of the treatment groups demonstrated tumor regression, as 100% of the mice demonstrated large primary tumors (Fig. 8, *A* and *B*) despite the presence of MMAC/PTEN expression in the tumors (Fig. 8*C*). Treatment with Ad-MMAC resulted in a 35% decrease in primary tumor mass as compared with treatment with PBS ($P < 0.05$; Fig. 5*C*). However, treatment with Ad-GFP resulted in a similar inhibition of tumor mass ($P = 0.78$ *versus* Ad-MMAC). This suggested that the observed reduction in tumor size by *in vivo* Ad-MMAC treatment was largely because of the toxicity of the multiple injections of adenovirus and not because of MMAC/PTEN expression. However, although *in vivo* treatment with Ad-MMAC did not have a significant effect on primary tumor growth, it markedly inhibited the formation of lymph node metastases (Figs. 5*D* and 8*B*). Although the presence of metastases in lymph nodes or at other sites was not examined, any enlarged nodes surrounding the primary tumor were examined for the presence of tumors. Lymph node metastases were grossly detected in 87% of PBS-injected tumors, 63% of Ad-GFP-treated tumors, and 25% of Ad-MMAC-treated tumors. These findings were confirmed by histological analysis. Thus, the *in vivo* treatment of pre-established tumors of PC3 cells with Ad-MMAC did not induce tumor regression or significantly reduce tumor size, but it did diminish the formation of metastases. To determine that the primary tumors that formed in Ad-MMAC-infected cells were not because of loss of expression of MMAC, Western blotting was performed after immunoprecipitation of tumor cell lysates with MMAC/PTEN-specific antibodies as described in "Materials and Methods." As is observed in Fig. 8*C*, primary tumors from Ad-MMAC-infected cells clearly demonstrate the presence of the *MMAC/PTEN* gene product. Thus, the lack of tumor regression and/or reduction in size of tumors cannot be attributed to loss of MMAC/PTEN expression.

DISCUSSION

In the studies reported here, we have examined the impact of adenovirus-mediated MMAC/PTEN expression on the behavior of human prostate cancer cells under both *in vitro* and *in vivo* conditions in the human prostate tumor cell line, PC3. The goal of the studies was to determine whether MMAC/PTEN inhibited the development of lymph node metastases in cells implanted orthotopically in the prostate tissue of nude mice. This model more closely mimics the biology of human prostate cancer than s.c. implantation, as previous studies have demonstrated that the tissue microenvironment of the mouse prostate promotes rapid growth of the human prostate cancer cells locally and the development of spontaneous metastases (32, 33).

First, the effects of ectopic expression of MMAC/PTEN on signaling pathways, proliferation, and survival were determined. Our results are consistent with the now well-established model in which MMAC/PTEN regulates the PI3K signaling pathway by decreasing the pools of 3′-phosphorylated phosphatidylinositol (reviewed in Ref. 43). In contrast, no effects of MMAC/PTEN expression on phosphorylation of FAK were observed. These results are similar to those observed in several tumor cell lines and in MMAC/PTEN$^{-/-}$ fibroblasts, in which increased phosphorylation of Akt/PKB, but no differences in FAK phosphorylation, are observed relative to *MMAC/PTEN*$^{+/+}$ cells. Thus, although multiple lines of evidence support the activity of

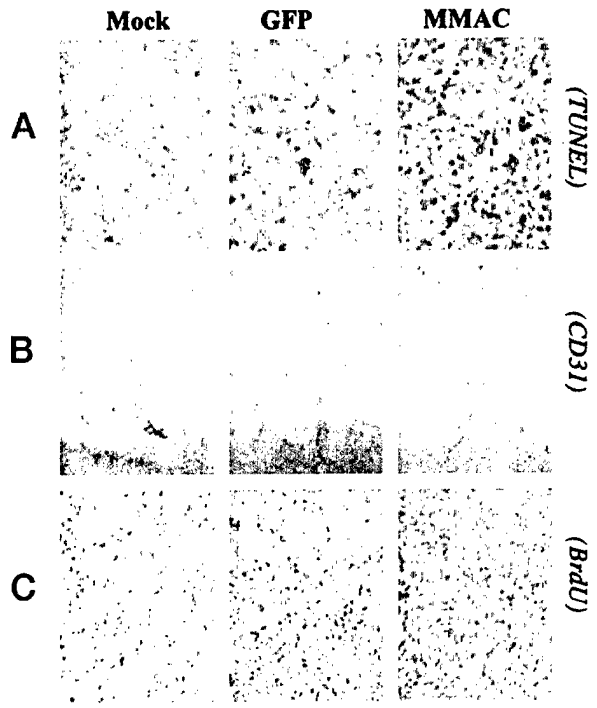

Fig. 7 Effect of *ex vivo* MMAC/PTEN expression on *in vivo* apoptosis, angiogenesis, and proliferation of orthotopically implanted PC3 cells. Immunohistochemical analysis of the same primary prostate tumors. *A*, TUNEL staining for apoptotic cells (*top row*, ×10 magnification). *B*, CD31 staining for endothelial cells, indicating vessel formation (*middle*, ×4 magnification). *C*, BrdUrd incorporation, indicating cellular proliferation (*bottom*, ×10 magnification). In each section, positively staining cells appear *red*. Sections shown are representative of the general appearance of individual tumors in multiple animals in each treatment group.

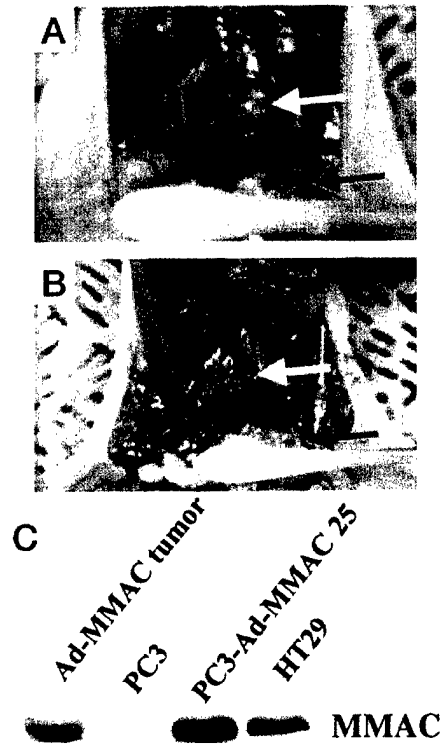

Fig. 8 PC3 tumors treated *in vivo* with Ad-GFP or Ad-MMAC. Photographs show representative gross anatomy of mice bearing PC3 tumors that were injected twice with Ad-GFP (*A*) or Ad-MMAC (*B*). *Green arrows*, the prostate tumors; *yellow arrows*, para-aortic lymph nodes. Prostate tumors treated with Ad-GFP demonstrated large, hardened lymph nodes as seen in *A*, which histological examination confirmed represented metastases of the primary prostate tumor. Tumors treated with Ad-MMAC showed small, soft lymph nodes that did not contain prostate tumors cell. *C*, expression of MMAC/PTEN. MMAC/PTEN was immunoprecipitated from 500 μg of lysates from mouse tumors (Ad-MMAC tumor) or cell lines (PC3, PC3 infected with Ad MMAC at 25 MOI, and HT29 cells, a colon tumor cell line expressing MMAC/PTEN). Western blotting was performed to detect MMAC/PTEN as described in "Materials and Methods."

MMAC/PTEN as a phosphatidylinositol phosphatase, the studies presented here do not support the identification of FAK as a substrate of MMAC/PTEN's putative protein phosphatase activity.

The expression of MMAC/PTEN in PC3 cells resulted in a significant inhibition of growth as compared with mock and control adenovirus treatments. This growth inhibition is not because of increased induction of apoptosis. These biological effects are similar to those we observed previously in experiments using the LNCaP human prostate cancer cell line (16). Although LNCaP cells demonstrate some apoptosis after high levels of MMAC/PTEN expression, quantitatively, the amount of apoptosis induced did not explain the extent of growth inhibition observed. Additionally, overexpression of Bcl-2 protein blocked MMAC/PTEN-induced apoptosis in LNCaP cells but not its growth inhibitory effects. The predominant regulation of prostate proliferation by MMAC/PTEN was also reported in the analyses of hyperplastic prostate tissue of *MMAC/PTEN*$^{+/-}$ mice and of prostate tumors in *MMAC/PTEN*$^{+/-}$ *Cdkn1b* +/− mice. In both of these studies, no changes in apoptotic indices were detected in the *MMAC/PTEN*$^{+/-}$ prostate tissues, but significant increases in proliferative indices were, as compared with *MMAC/PTEN*$^{+/+}$ mice (28).

The tumor suppressor gene *p53* is also inactivated frequently in metastatic prostate cancer (2, 44–47) and is currently being used in clinical trials in prostate cancer patients (48). In the studies in LNCaP cells, the expression of MMAC/PTEN was significantly more growth inhibitory than ectopic expression of p53. However, in the studies presented here, p53 expression inhibited the growth and survival of PC3 cells significantly more than the expression of MMAC/PTEN. The extent of growth inhibition induced by p53 observed here is very consistent with earlier published reports (31). The difference in comparative sensitivities to these two treatments between these two prostate cell lines could be because of a number of factors. The most likely explanation for this difference is that LNCaP cells harbor wild-type p53 function, whereas PC3 cells do not. Thus, p53 would be expected to be more growth inhibitory in the latter cells. A similar pattern of differing sensitivity to MMAC/PTEN and p53 has also been observed in human glioma cell lines. We reported previously that the expression of MMAC/PTEN in the

U251 human glioma cell line, which harbors mutant p53, resulted in a minimal inhibition of growth, whereas p53 expression potently inhibited U251 growth and survival (16, 36). In contrast, the U87 human glioma cell line, which retains wild-type p53 function, is more sensitive to adenoviral MMAC/PTEN expression than to adenoviral p53 (34). Paramio *et al.* (49) have demonstrated that MMAC/PTEN-mediated growth inhibition is not p53 dependent. Although these experiments were not conducted in prostate cells, MMAC/PTEN expression was growth inhibitory to PC3 cells in these experiments, suggesting that MMAC/PTEN-induced growth inhibition in prostate cells also is not p53 dependent. Therefore, there appears to be no direct relationship between the status of p53 mutation and sensitivity to ectopic expression of MMAC/PTEN.

Two previous studies have examined the effects of MMAC/PTEN expression in PC3 cells *in vitro*. Similar to this report, Sharrard *et al.* (50) observed an inhibition of PC3 cell growth after MMAC/PTEN expression without an induction of apoptosis. In contrast, Persad *et al.* (51) reported that the expression of MMAC/PTEN in PC3 cells resulted overwhelmingly in the induction of apoptosis. However, apoptosis was demonstrated only in cells that were maintained in serum-free media for 48 h. Thus, these results do not contradict the results obtained here, as we examined cells maintained in mitogen-enriched tissue culture media. It is clear by the Western blotting presented in this report (Fig. 2) that the mitogenic pathways of PC3 cells are sensitive to the mitogen content of the media, as cells starved for only 18 h demonstrated a decrease in Akt/PKB phosphorylation. Thus, in the studies of Persad *et al.* (51), the expression of MMAC/PTEN in addition to serum starvation may have inhibited this prosurvival pathway sufficiently to induce the apoptotic cascade.

***In Vivo* Studies.** Having established that ectopic expression of MMAC/PTEN in PC3 cells resulted in biological and signaling effects similar to those reported for many tumor cell lines, the main goal of this study was to examine the *in vivo* function of MMAC/PTEN in human prostate cancer cells. Two different experimental approaches were used. In the first experiments described, MMAC/PTEN expression was introduced *ex vivo* by treating the cells with Ad-MMAC before implanting the cells in nude mice. This technique has the advantage of allowing uniform gene expression with a single treatment of the cells and involves minimal mechanical toxicity. However, it is a somewhat artificial system to evaluate a gene for potential therapy applications, as treatment of patients will involve expressing the gene in tumor cells already present in the prostate. Therefore, the effects of *in vivo* treatment of pre-established PC3 tumors with Ad-MMAC were also examined. This allows for a more relevant examination of the potential for clinical gene therapy. However, this technique produces more heterogeneous and limited gene expression in the tumor cells and intrinsically involves more mechanical trauma to the tumor. Thus, the *in vivo* expression technique may be somewhat limited in accurately representing the true *in vivo* function of the gene.

The *ex vivo* expression of MMAC/PTEN in PC3 cells did not inhibit the tumorigenicity of these cells when they were implanted orthotopically. However, *ex vivo* expression of MMAC/PTEN did significantly reduce local tumor size by inhibiting cellular proliferation, a result that is consistent with the *in vitro* experiments. Additionally, *ex vivo* expression of MMAC/PTEN completely inhibited the development of lymph node metastases by PC3 cells. Thus, these experiments suggest that MMAC/PTEN is not a critical regulator of prostate tumor formation but is a critical regulator of aggressive local prostate tumor growth and metastasis. This model is consistent with the pattern of mutations and deletions of *MMAC/PTEN* that have been detected in human prostate cancer clinical specimens but represents the first *in vivo* demonstration of this proposed role. However, this finding does contrast with the observation in genetically engineered mice that loss of a single *MMAC/PTEN* allele promotes prostate hyperplasia and tumor formation (27). Although those murine lesions and tumors may have also included other genetic alterations not recapitulated in the PC3 cell line, it is notable that there is no report of an increased incidence of abnormal prostate proliferation in the familial syndromes that harbor germ-line *MMAC/PTEN* mutations. Thus, the results in the genetically engineered mice may reflect a different regulation of prostate homeostasis in that species than is functional in the human tissue.

The *in vivo* treatment of pre-established tumors of PC3 cells with Ad-MMAC did not induce tumor regression but did moderately inhibit localized prostate tumor size. This reduction in tumor size could not be attributed to MMAC/PTEN expression, however, as infection with a control adenovirus resulted in a similar degree of inhibition. Substantial variation in the size of the tumors, particularly in the Ad-MMAC treatment group, indicates that repeated injections into the prostate tumors resulted in nonspecific toxicity. Furthermore, it is expected that not all tumor cells will be infected by this procedure. Thus, in this experimental setting, it is difficult to assess the effect of MMAC/PTEN expression on the local growth of the cancer cells. However, *in vivo* treatment of these tumors with Ad-MMAC did have an inhibitory effect on the metastatic behavior of these cells. This effect was apparent in the rate of lymph node metastasis, which was confirmed by histological analysis. The potent inhibition of the metastasis of PC3 cells by MMAC/PTEN is consistent with the effects of *ex vivo* expression and, again, with the detected pattern of genetic inactivation of *MMAC/PTEN* in clinical specimens.

The inhibition of PC3 prostate cancer cell metastasis after both *ex vivo* and *in vivo* expression of MMAC/PTEN is striking. Although these studies did not determine the specific mechanism of this effect; previous studies have demonstrated that MMAC/PTEN can regulate a number of *in vitro* behaviors characteristic of metastatic cancer cells. These regulated behaviors include anoikis, motility, and invasion (reviewed in Ref. 13). Although MMAC/PTEN expression clearly regulates signaling pathways responsible for the above biological behaviors, it is also possible that MMAC/PTEN's inhibitory effect on metastasis may be because of the regulation of factors released into the circulation by the tumor cells in the prostate. This hypothesis should also be investigated, as Ad-MMAC treatment of pre-established tumors likely resulted in heterogeneous expression of MMAC/PTEN but still inhibited the formation of metastases.

The effects of adenoviral p53 on the *in vivo* growth of PC3 cells have also been investigated previously. Two separate reports have demonstrated that the *ex vivo* expression of p53 in

PC3 cells inhibits the tumorigenicity of these cells (52, 53). More recently, Eastham *et al.* (31) have reported that a single injection of adenoviral p53 into pre-established orthotopic PC3 tumors resulted in the complete regression of several tumors, a 75% reduction in local tumor size, and a 55% reduction in metastasis formation. Although different numbers of cells and amounts of adenovirus were used, the reported increased *in vivo* growth-suppressive effects of p53 expression in PC3 cells as compared with MMAC/PTEN agree with the increased inhibitory effect that we observed *in vitro*. Although it was not determined *in vivo*, each of these studies demonstrated clear evidence *in vitro* of a significant induction of apoptosis after p53 expression. Given that MMAC/PTEN-mediated growth inhibition does not seem to be predominantly mediated by apoptosis, a combined gene therapy of MMAC/PTEN and p53 might provide an additive effect.

As yet, few studies have examined the effects of *in vivo* treatment of pre-established tumors with adenoviral MMAC/PTEN. Sakurada *et al.* (54) examined the effects of MMAC/PTEN gene therapy on pre-established tumors of human endometrial cancer cells and reported a cytostatic effect. However, the cells were injected s.c. into the mice instead of orthotopically, and the effects of MMAC/PTEN expression on metastasis formation were not reported. Recently, Wen *et al.* (55) reported the effects of stable *ex vivo* expression of MMAC/PTEN on the growth of orthotopically implanted U87 human glioma cells. Similar to the results reported here in PC3 cells, MMAC/PTEN expression did not inhibit the tumorigenicity of the U87 cells but did reduce tumor size and prolonged survival.

In summary, the results presented here functionally support the proposed role of MMAC/PTEN as a regulator of prostate cancer progression. Specifically, they have demonstrated that MMAC/PTEN inhibits the metastatic potential of PC3 cells without inhibiting local prostate tumor formation. These studies also suggest that additional investigations are indicated to assess MMAC/PTEN's potential as a prognostic marker for staging and treatment purposes and also as a potential therapeutic tool.

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A Urokinase-Derived Peptide (Å6) Increases Survival of Mice Bearing Orthotopically Grown Prostate Cancer and Reduces Lymph Node Metastasis

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The high rate of prostate cancer mortality invariably reflects the inability to control the spread of the disease. The urokinase-type plasminogen activator and its receptor (u-PAR) contribute to prostate cancer metastases by promoting extracellular matrix degradation and growth factor activation. The current study was undertaken to determine the efficacy of a urokinase-derived peptide (Å6) in reducing the lymph node metastases of prostate cancer using a model in which prostatic tumors established in nude mice from orthotopically implanted PC-3 LN4 prostate cancer cells disseminate to the lymph nodes. As a first step in evaluating the *in vivo* effectiveness of Å6, we determined its effect on *in vitro* invasiveness. *In vitro*, Å6 reduced the invasiveness of PC-3 LN4 cells through a Matrigel-coated filter without affecting growth rate. A first *in vivo* survival experiment showed that all Å6-treated mice were alive after 57 days, and half of them tumor-free, whereas all control mice receiving vehicle had died. In a second experiment with a larger tumor inoculum and a longer delay until treatment, whereas 71% of control mice and 83% of mice treated with a scrambled peptide developed lymph node metastases, only 22 to 25% of Å6-treated mice had positive lymph nodes. Further, lymph node volume, reflective of tumor burden at the secondary site, was diminished 70% in Å6-treated mice. In conclusion, we provide definitive evidence that a peptide spanning the connecting region of urokinase suppresses metastases and, as a single modality, prolongs the life span of prostate tumor-bearing mice. *(Am J Pathol 2003, 162:619–626)*

Prostate cancer afflicts 209,000 American men every year and is second only to lung cancer as the leading cause of cancer deaths in the male population. The high rate of mortality invariably reflects spread of this disease to the secondary sites and consequently, effective treatments in the future will require a means of combating prostate cancer dissemination.

It is well established that the spread of virtually all malignancies require the expression of one or more proteases which serve to cleave extracellular matrix and activate growth factors.[1] The urokinase-type plasminogen activator [2] contributes to tumor progression by converting plasminogen into plasmin a widely acting serine protease that cleaves several basement membrane components including laminin and fibronectin[3] as well as type IV collagen indirectly *via* activation of metalloproteinases.[4] Urokinase, achieves this by binding to a cell surface receptor (u-PAR)[5,6] which increases the efficiency by which plasminogen is converted into plasmin.[7] Further, urokinase cleaves u-PAR thereby promoting chemotaxis.[8,9]

There is currently strong evidence implicating the urokinase-u-PAR axis in prostate cancer progression. For example, the urokinase gene is amplified in some hormone-refractory prostate cancers[10] and overexpression of this protease increases skeletal metastases of this malignancy.[11] Additionally, in two separate studies,[12,13] the expression of an exogenous plasmid encoding urokinase lacking an enzyme active site, prevented metastases of human (PC-3) and murine (MAT-LyLu) prostate cancers. Further, independent studies by Festuccia et al[14] and Hollas et al[15] reported that urokinase-u-PAR complexes characterized the invasive phenotype of cultured prostate cancer cells and that antibodies that prevented this interaction blocked *in vitro* invasion. Moreover, high u-PAR levels in the serum is predictive of metastatic prostate cancer and shortened survival time.[16] Taken together, these reports would suggest that the urokinase-u-PAR axis represents a therapeutic target for controlling prostate cancer metastases. We therefore determined the potential of a urokinase-derived peptide (Ac-KPSSPPEE-amide, hereafter referred to as Å6) spanning amino acids 136–143 to counter the metastases of orthotopically grown prostate cancer. This peptide, which non-competitively blocks the interaction of urokinase with

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soluble u-PAR *in vitro*,[17] has proven efficacious in reducing glioblastoma growth and angiogenesis.[18]

Materials and Methods

Cell Culture

Establishment of the highly metastatic PC-3 LN4 cell line has been described elsewhere.[19] Cells were grown in DMEM/F12 culture medium containing 10% FBS.

Western Blotting for u-PAR

Western blotting for u-PAR was performed as described previously.[20] Briefly, cells were extracted into a Triton X-100-containing buffer supplemented with protease inhibitors. Insoluble material was removed by centrifugation and the cell extract immunoprecipitated with a polyclonal anti-u-PAR antibody. The immunoprecipitated material was then subjected to standard Western blotting and the blot probed with 5 μg/ml of an anti-u-PAR monoclonal antibody (no. 3931, American Diagnostica, Greenwich, CT) and an HRP-conjugated goat anti-mouse IgG. Bands were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Northern Blotting

The level of steady state mRNAs was determined by Northern analysis.[20] Total cellular RNA was extracted from 90% confluent cultures using 5.0 mol/L guanidinium isothiocyanate and purified on a cesium chloride cushion (5.7 mol/L) by centrifugation at 150,000 × *g* for 20 hours. Purified RNA was electrophoresed in a 1.5% agarose-formaldehyde gel and transferred to Nytran-modified nylon by capillary action using 10X SSC. The Northern blot was probed at 42°C with a random primed radiolabeled u-PAR cDNA which starts at the transcription start site and extends 0.65 kb downstream. The blots were then washed at 65°C using 0.25X SSC in the presence of 0.75% SDS.

In Vitro *Invasion Assays*

These were performed as described by this laboratory previously,[21] but with modifications. Briefly, cells are dispersed with 4 mmol/L EDTA and 250,000 cells dispensed into a BD BioCoat Falcon cell culture chamber (BD Biosciences Bedford, MA). The chamber was subsequently inserted into the outer well, the latter also containing culture medium. The cells were incubated at 37°C for 2 days after which the cells on the upper aspect of the filter were removed with a cotton swab and cells on the lower aspect stained using the DifQuik kit. Invaded cells were enumerated.

Orthotopic Model to Assess Prolongation of Life Span

Nu/Nu mice (8–12 weeks of age) were injected with 2 × 10^5 PC-3 LN4 cells/50 μl in Ca^{2+}, Mg^{2+}-free HBSS into the prostate as described previously.[19] After 3 days to allow for tumor establishment, mice were injected every 12 hours with either vehicle (PBS) or Å6. Each Å6 injection contained 37.5 mg/kg such that the daily dose was 75 mg/kg.

Orthotopic Model for Measuring in Vivo *Metastases*

These were carried out as above, except that the tumor inoculum was 5 × 10^5 PC-3 LN4 cells. After 7 days, when primary tumors were palpable, Å6 or Å16 was administered i.p. twice daily at 25 or 75 mg/kg/day body weight. In the male mouse, the prostate is located in the bladder

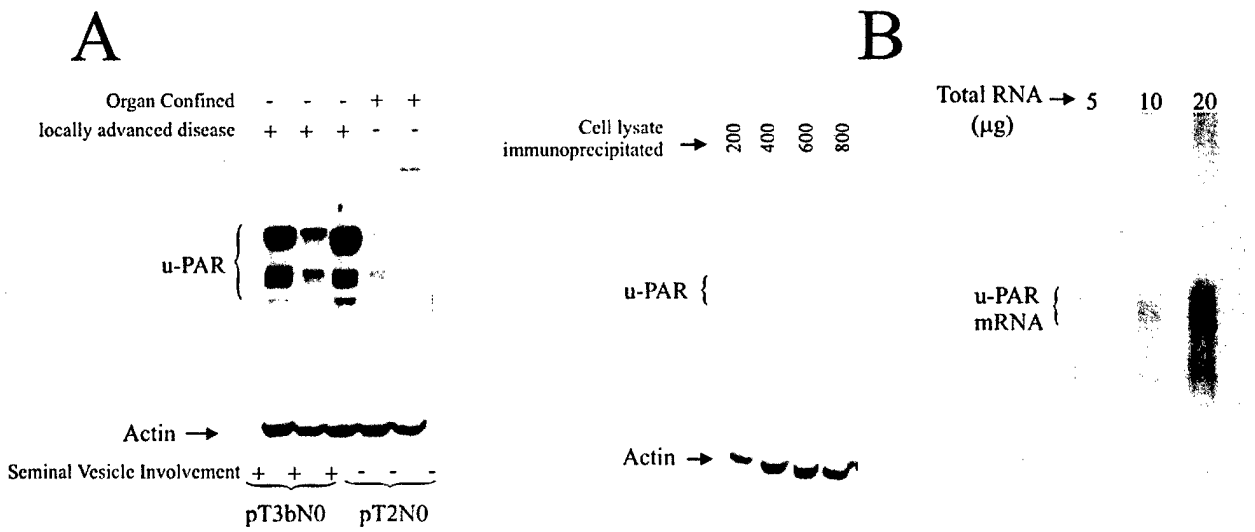


Figure 1. u-PAR expression is increased in locally advanced prostate cancer. **A:** Equal protein (500 μg) from resected prostate tumor extracts was immunoprecipitated with an anti-u-PAR antibody and analyzed for u-PAR protein by Western blotting. The residual supernatant from the immunoprecipitated was immunoblotted for actin. **B:** PC-3 LN4 cells were analyzed for u-PAR protein (**left panel**) or for mRNA (**right panel**). Western blotting was as described for **panel A** with the exception that the amount of protein immunoprecipitated was varied. For the Northern blotting, total RNA (20 μg) from PC-3 LN4 cells was subjected to Northern blotting for u-PAR mRNA.

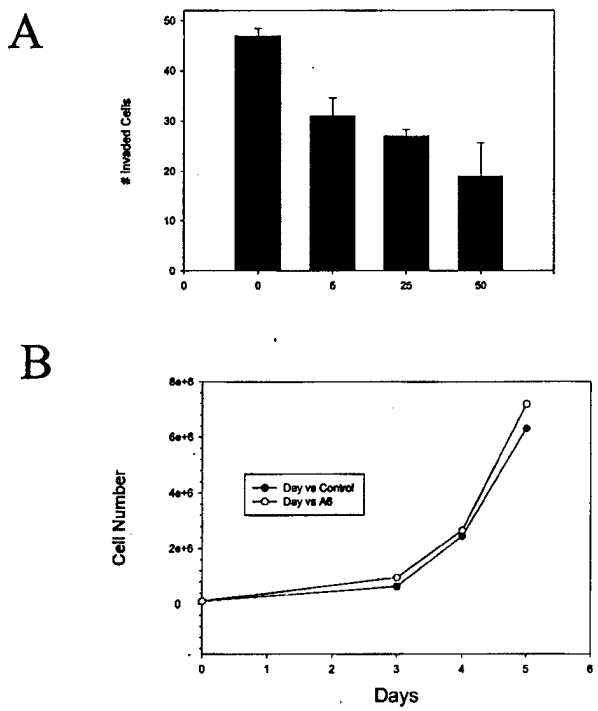


Figure 2. Å6 reduces *in vitro* invasiveness of PC-3 LN4 cells. **A:** PC-3 LN4 (2.5×10^5 cells) were plated on Matrigel-coated porous filters. After 6 hours to allow for cell attachment, Å6, was added at the indicated concentration. Two days later, the cells on the upper aspect of the filter were removed and invaded cells stained, photographed and then enumerated. Data are presented as mean ± SD of three independent assays. **B:** Growth curve of PC-3 LN4 grown in the presence, or absence, of 50 μmol/L Å6.

neck (outlet), allowing for easy palpation of an enlarged prostatic mass from the outside.[22] The prostate mass is round, fixed and hard. At the end of the experiment, mice were sacrificed and prostate and lymph nodes examined both macroscopically and microscopically for the presence of tumor cells. For histological examination, tissues were stained with hematoxylin and eosin.

Statistics

Statistical analysis was performed using the Instat (version 3.05) and Prism statistical software (GraphPad, San Diego, CA). The Mann-Whitney non-parametric test (two-tailed) was used to test for statistical significant differences between Å6-treated and untreated cells in the *in vitro* invasion assays as well as reductions in lymph node volumes in the *in vivo* model. Survival curves were tested for statistically significant differences using the log rank test.

Results

Elevated u-PAR Expression in Locally Advanced Prostatic Cancer

Previous studies with prostate cancer have strongly suggested a role for the urokinase-u-PAR axis in the progression of this disease. Therefore, u-PAR protein levels were compared in resected prostate cancer derived from patients with organ confined or locally advanced (demonstrating seminal vesicle involvement) disease. All tumors were resected at the M.D. Anderson Cancer Center. Three tumors had bilateral extracapsular tumor extension without regional lymph node metastases (pT3bN0) while two other tumors were palpable but confined to the prostate (pT2N0). While u-PAR protein was abundant in tumor extracts from three patients with locally advanced disease (Figure 1A), the amount of this protein derived from patients with organ-confined disease was at, or below, the detection limit of Western blotting. These results are consistent with previous studies demonstrating that the urokinase/u-PAR axis contributes to prostate tumor progression, and that inhibition of this proteolytic axis might limit this process.

Reduction of in Vitro *PC-3 LN4 Invasion by Å6*

We first determined the ability of Å6 to diminish *in vitro* invasiveness of PC-3 LN4 cells. These cells express both u-PAR as evidenced by both Northern and Western blotting (Figure 1B) and urokinase as shown previously.[14,15] Cells were plated on Matrigel-coated porous filters and after 6 hours to allow for cell attachment, Å6 added at varying concentrations shown previously to reduce *in vitro* breast cancer invasiveness.[17] In the absence of the peptide, PC-3 LN4 showed a pronounced invasiveness through the extracellular matrix-coated barrier (Figure 2A). However, addition of the urokinase-derived peptide resulted in a dose-dependent decrease in the number of cells traversing the Matrigel-coated filter (Figure 2A). At 50 μmol/L, Å6 caused about a 60% diminution in *in vitro* invasion by PC-3 LN4 cells. This difference was statistically significant ($P < 0.05$). The reduced invasiveness

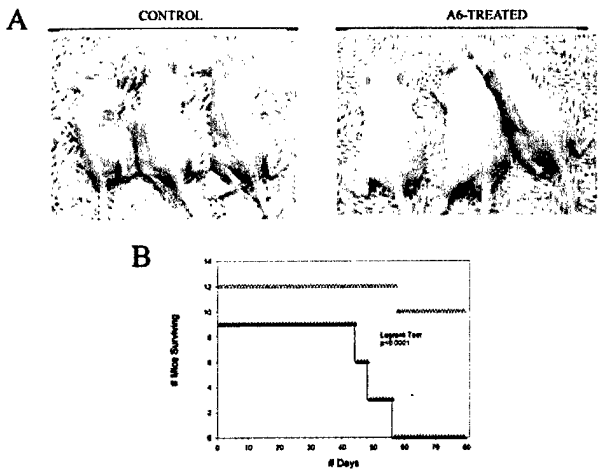
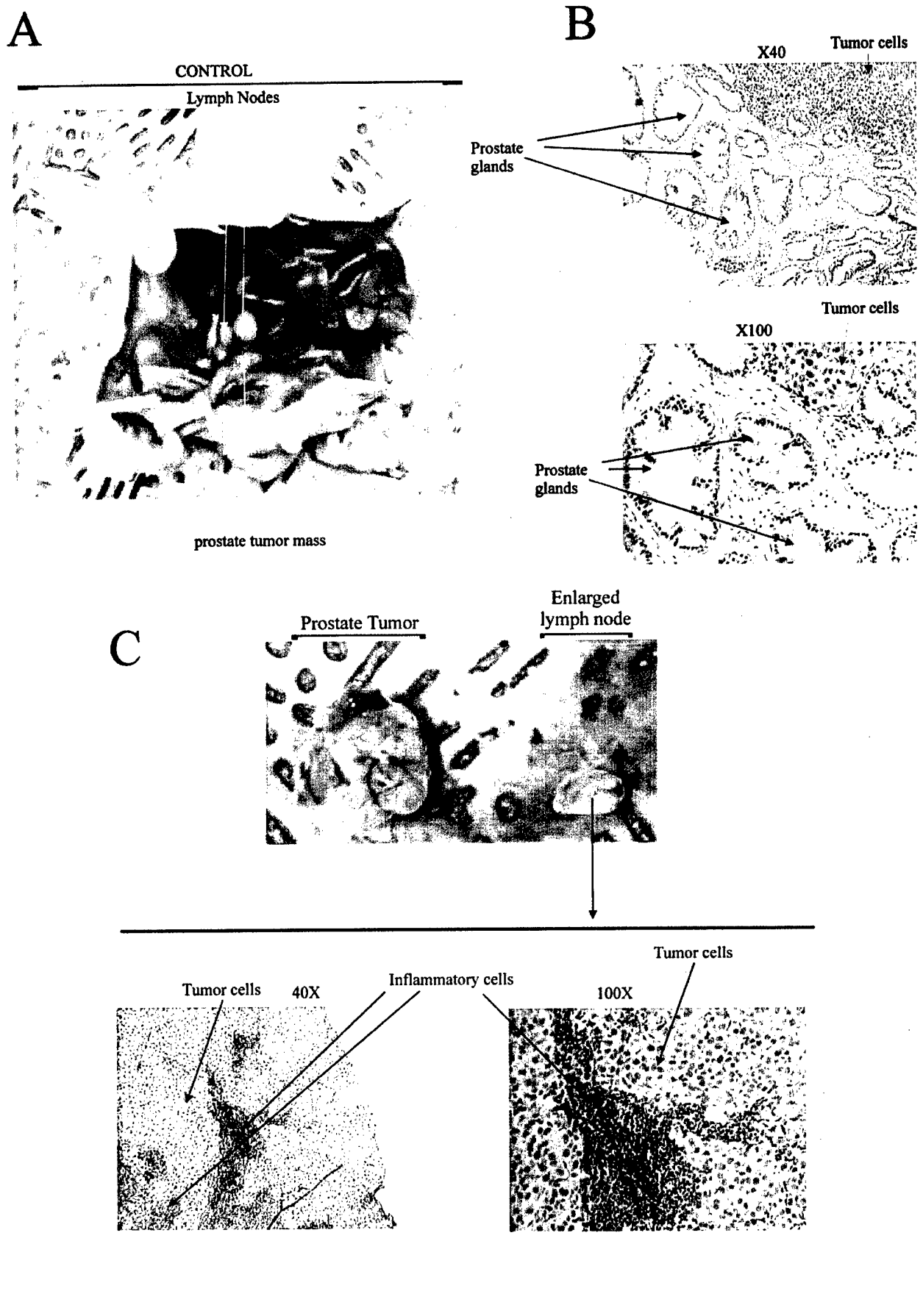


Figure 3. Untreated, but not Å6-treated, PC-3 LN4-bearing mice show severe cachexia and decreased survival. **A:** Pictures of mice bearing orthotopically grown PC-3 LN4 tumors after daily treatment for 50 days with carrier (PBS-control) or 75 mg/kg Å6. **B:** Mice were orthotopically injected with 2×10^5 cells. When primary tumors were palpable, mice were divided into two groups with one group receiving PBS alone and the other administered with 75 mg/kg Å6 on a daily basis. Mice were sacrificed when moribund as determined by standard IACUC criteria. Differences in the survival rates were tested for statistical significance using the log rank test.


A
CONTROL
Lymph Nodes
prostate tumor mass
B
X40
Tumor cells
Prostate glands
Tumor cells
X100
Prostate glands
C
Prostate Tumor
Enlarged lymph node
Tumor cells
40X
Inflammatory cells
Tumor cells
100X

was not due to a diminished cell proliferation (Figure 2B). The diminished invasion of PC-3 LN4 cells was less impressive than previously published for the urokinase inhibitor amiloride.[10] It may be that the greater effect with amiloride reflects its multiple mechanisms of action. Indeed, in addition to inhibiting urokinase activity,[23] amiloride also reduces mRNA levels for this plasminogen activator and its receptor.[24,25]

Å6 Prolongs the Survival Time of PC-3 LN4-Bearing Mice

To assess the efficacy of Å6 in prolonging life span, we used an *in vivo* model in which 2×10^5 highly metastatic PC-3 LN4 cells are injected orthotopically into the prostate. After 3 days to allow for tumor establishment, mice were injected every 12 hours with either vehicle (PBS) or Å6 (75 mg/kg/day). After 38 days, all of the mice in the control group had palpable prostatic tumors whereas none of the mice in the Å6-treated group had palpable tumors. The severe cachexia demonstrated by the control group, but not by the Å6-treated mice, is apparent at day 50 (Figure 3A). The control mice were sacrificed when moribund (Figure 3B) to keep the study within IACUC guidelines. While there were 2 deaths in the Å6-treated mice after 59 days (Figure 3B), these deaths were not due to tumor, since at autopsy, both the prostate and lymph nodes were observed to be tumor-free. Deaths in these Å6-treated mice were due to systemic infection originating at the injection site as a consequence of inadequate sterile technique compounded by the frequent injection schedule. The surviving Å6-treated mice were sacrificed at day 80 and autopsied. Of these, 4 of 10 mice were deemed to be tumor-free. Of the 6 mice that developed primary tumors, only 2 showed lymph nodes positive for disease. It is noteworthy that the reduced incidence (50%) of primary tumor development in the Å6-treated mice compares with 100% tumor development in mice receiving the vehicle alone. Log rank analysis indicated that the prolonged survival of the Å6-treated mice compared with the controls was statistically significant ($P < 0.0001$).

Å6 Attenuates the Metastasis of Prostate Cancer in an Orthotopic Model

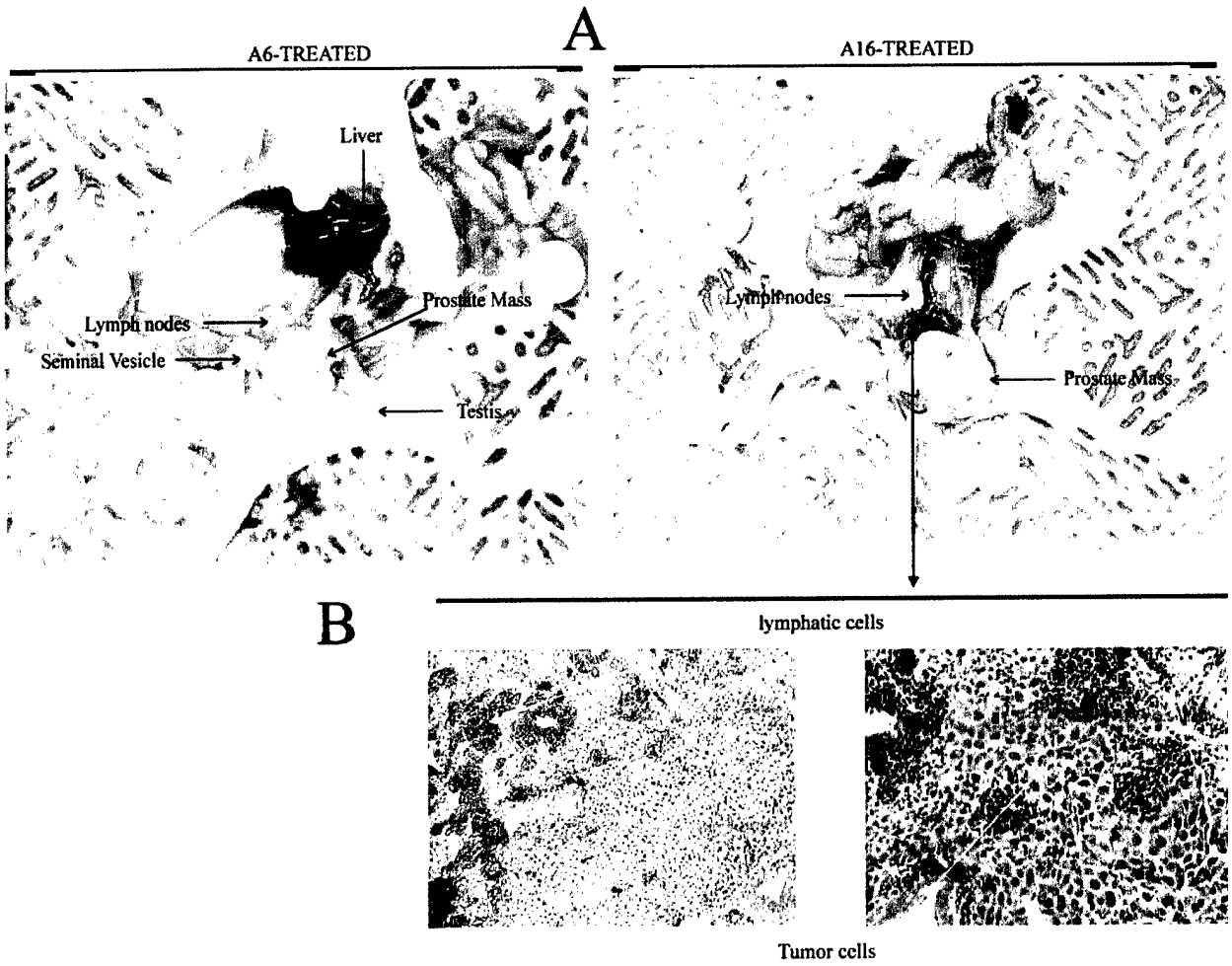
Since Å6 effectively prevented the establishment of primary prostatic tumors in the model described above, it was not possible to assess the drug's activity against their metastasis. Therefore, a second study was conducted using the above model, but, with a larger tumor inoculum and withholding treatment for 7 days until the primary tumor mass was definitely palpable. Inoculation of 5×10^5 PC-3 LN4 cells, with no treatment, resulted in the formation of an enlarged prostatic tumor mass at the primary site (Figure 4, A and B) at a rate of between 85 and 100% after 6 weeks. The majority of these mice (71%) demonstrated spread of the disease to the regional lymph nodes as evidenced both macroscopically (Figure 4A) and microscopically (Figure 4C). In the treated group, mice were injected twice daily with 25 or 75 mg/kg/day of Å6 or as a control, Å16, a peptide comprised of the scrambled Å6 amino acid sequence (Ac-PSESPEKP-NH_2). After 6 weeks, mice were sacrificed and tumor weights and lymph node involvement determined. Å6 treatment had little effect on primary tumor mass (Figure 5, A and C). However, Å6, but not the control peptide Å16, reduced the percentage of mice with tumor cell-positive lymph nodes from 71 to as low as 22% (Figure 5, B and C). Further, while Å6 diminished lymph node volume (Figure 5, A–C) by up to 70% ($P = 0.004$), the control peptide, Å16, had no effect on this parameter.

Discussion

Effective therapy of prostate cancer is hampered by the lack of suitable agents for controlling the spread of the disease. Indeed the poor prognosis of those patients who present with metastatic disease compared with those individuals with organ-confined prostate cancer (81% and 25% disease-free at 10 years, respectively) indicate the urgent need for strategies to combat the metastatic disease. We demonstrate, herein, the efficacy of Å6, a urokinase-derived peptide previously shown to interfere with the urokinase-u-PAR system,[17] in reducing the metastatic spread of orthotopically grown prostate cancer and prolonging the life span of prostate cancer-bearing mice.

Å6 has previously been shown to have anti-tumor effects on other experimental cancers including mammary adenocarcinoma[17,26] and glioblastoma.[18] However, while the previous study of Guo and co-workers[17] indicated an anti-metastatic effect of Å6, since the primary tumor mass was reduced in both cases, it was difficult to determine whether reduced metastases was a direct effect of the agent on the metastatic process or secondary to a smaller primary tumor size. Our study was intended to resolve this issue and determine whether Å6 has an anti-metastatic effect. In our first experiment with the PC-3 LN4 highly metastatic orthotopic prostatic carcinoma model that used treatment 3 days after a tumor inoculum of 2×10^5 cells, we fully expected the primary tumor to establish itself and metastasize despite treatment with Å6. It was therefore a surprise to note that the treated animals fared very well compared to the untreated controls, and we therefore extended the duration of treatment of 10 mice to 80 days. It is noteworthy that 4 of these 10 mice were tumor-free on autopsy. Of the 6 mice that did have a primary tumor, only 2 had lymph

Figure 4. Extra-prostatic spread of orthotopically grown PC-3 LN4 to the lymph nodes. PC-3 LN4 cells (5×10^5) were injected into the prostate of male nu/nu mice. After 6 weeks, mice were sacrificed and prostate and lymph node tissues fixed and stained for histological examination. **A** depicts the prostate tumor mass and enlarged lymph nodes while histological evidence of tumor cells in the prostatic mass are illustrated in **B**. The presence of tumor cells in the enlarged lymph nodes is apparent by histology (**C**). These data confirm the spread of the orthotopically implanted prostate cancer cells to the lymph nodes.



C

				Lymph Node Volume (cm^3)		
	# Mice	Mean Primary Tumor Mass (g)	% Mice with Positive Lymph Nodes	Average	Range	Probability
Control	8	0.58	71	7.1	0-56	
Å6 (25 mg/kg)	8	0.44	25	2.2	0-6	p =0.004
Å6 (75 mg/kg)	7	0.49	22	2.4	0-6	p=0.05
A16 (75 mg/kg)	6	0.7	83	9.6	0-19	p=0.146

Figure 5. Å6 reduces the lymph node-spread of orthotopically grown PC-3 LN4 cells. **A, B:** PC-3 LN4 cells (5×10^5 cells) were injected into the prostates of nu/nu mice. When primary tumors were established, as determined by palpation, mice were injected twice daily with the indicated doses of Å6 or Å16. After 6 weeks, mice were sacrificed, and examined macroscopically and microscopically for spread of the prostate cancer to the lymph nodes. **C:** Statistical analysis of the effect of Å6 and Å16 on the spread of PC-3 LN4 to the lymph nodes. Probability levels are given with respect to the control.

node metastases. This result was in pronounced contrast to the result from the untreated control animals, all of which succumbed to metastatic disease and exhibited severe cachexia before their sacrifice. These findings are consistent with other studies demonstrating an attenuating effect of Å6 on primary tumor volume.[18,26] In a second experiment, we therefore made a determined effort to establish the primary tumor before start of treatment. This was achieved with a larger tumor inoculum (5×10^5 cells) and waiting until the primary tumor was clearly palpable; this took 7 days. Å6 treatment started on day 8, continued for 6 weeks, and resulted in little effect on the primary tumor mass relative to controls but a marked effect on lymph node metastasis.

Thus, our findings are the first to show that Å6 administered alone effectively prolongs the survival of tumor-bearing mice, in this case orthotopically grown prostate cancer. We can only speculate as to how Å6 prolongs survival of the tumor-bearing mice. Certainly, in the clinical situation in humans, tumor progression is generally associated with protein wasting (negative nitrogen balance) and reduced food intake, all events that are self-reinforcing, thereby hastening the demise of the individual. Additionally, renal failure and bilateral ureter obstruction from lymph node enlargement and hepatic failure from spread of the tumor cells to the liver may also contribute to the increased morbidity of the control animals.

Our results contrast with a study[18] where it was reported that Å6 combined with cisplatin, but not as a single modality, increased survival of glioblastoma-bearing mice. Second, our findings demonstrate that in the second, more difficult to treat, model the effect of inhibiting the urokinase-u-PAR axis is entirely on the development of lymph node metastases and not growth of tumor at the primary site. A third, broad, conclusion that can be drawn by comparison of the two experiments is that earlier treatment with Å6 on smaller PC-3 LN4 tumors was much more effective than later treatment on larger tumors. This may have implications for how Å6 may best be used in cancer patients.

At the present time, there are few studies to identify candidate metastases-suppressing drugs. One exception is a previous report by Rabbani and colleagues,[27] who demonstrated the ability of the nucleoside analogue β-L-(−)dioxolane-cytidine to reduce the incidence and spread of the highly metastatic Dunning R3227 Mat Ly Lu cells to the adrenals. However, as in previous studies with Å6, it was not clear whether the benefit of this agent was due to its effect on the metastatic process or secondary to the marked decrease in tumor volume.

Considering the role of the urokinase-u-PAR axis in promoting extracellular matrix degradation, cell migration and chemotaxis[8,21,28] as well as preventing tumor dormancy,[29,30] antagonism of the urokinase-u-PAR axis represents one potential means of controlling spread of cancer. Some evidence for this possibility has been derived from the study of various malignancies including prostate cancer. In one study, the epidermal growth factor domain of murine urokinase fused to the Fc portion of human IgG proved to be a high affinity antagonist of the murine u-PAR and inhibited neovascularization and growth of B16 melanoma in syngeneic mice.[31] In another study, Goodson and others isolated a family of u-PAR-binding ligands by affinity selection of a 15-mer random peptide library displayed on bacteriophage M13.[32] Further, small molecular weight inhibitors of urokinase such as amiloride and *p*-aminobenzamidine[33] have proven effective in reducing LNCaP and DU145 prostate tumor growth in mice. While it is not possible at the present time to determine which of these agents would prove most efficacious in blocking the urokinase-u-PAR axis, one practical benefit of Å6 is its high aqueous solubility compared with the bacteriophage peptides isolated previously.[32]

The mechanism by which Å6 exerts its anti-tumor and anti-metastatic effects is presently unclear. In a previous study,[17] it was shown that Å6, which is derived from the connecting peptide of urokinase (amino acids 136–143) acts as a non-competitive antagonist of the u-PAR using a non-cell based biochemical assay in which soluble u-PAR and immobilized urokinase were used. However, it is also possible that Å6 mediates its anti-metastatic effects via other mechanisms. For example, phosphorylation of urokinase at serine 138 has previously been shown to modulate cell adhesion and motility[34] and Å6 might act as a phosphorylation substrate in competition with endogenous urokinase.

In conclusion, we have demonstrated the ability of Å6, a urokinase-derived peptide, to increase the survival of mice bearing orthotopically grown prostate cancer and to reduce the metastatic spread of this cancer to lymph nodes. Å6 can be added to a short list of agents that perturb the urokinase-u-PAR axis to counter tumor invasiveness. Our study sets the stage for future investigations to determine the clinical utility of Å6, or congeners, in reducing prostate cancer dissemination.

Acknowledgments

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Featured Article

Reduced c-Met Expression by an Adenovirus Expressing a c-Met Ribozyme Inhibits Tumorigenic Growth and Lymph Node Metastases of PC3-LN4 Prostate Tumor Cells in an Orthotopic Nude Mouse Model

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Abstract

Purpose: **The expression of c-Met, the receptor protein tyrosine kinase for hepatocyte growth factor/scatter factor, frequently increases during prostate tumor progression. However, whether reduced c-Met expression inhibits tumor growth and metastasis has not been ascertained.**

Experimental Design: **c-Met expression was reduced by infection of an adenovirus expressing a c-Met ribozyme into the highly metastatic human prostate cancer cell line PC3-LN4. *In vitro*, effects on c-Met, Akt, and extracellular signal-regulated kinase 1/2 expression and phosphorylation, Src expression and activity, and vascular endothelial growth factor expression were determined, as were effects on cell migration and invasion. Prostate tumor formation and metastasis to regional lymph nodes in nude mice were examined after both *ex vivo* and *in vivo* infection of cells.**

Results: **Infection of PC3-LN4 cells with the Ad-c-Met-expressing ribozyme decreased steady-state c-Met levels, decreased Src kinase activity, decreased vascular endothelial growth factor expression, and decreased migration and invasion *versus* the pU1 (control) virus. Significant inhibition of tumorigenicity (histologically confirmed tumors in only 1 of 10 mice) and consequent lymph node metastasis were observed upon *ex vivo* infection of Ad-c-Met. Similarly, gene therapy experiments led to complete inhibition of tumor growth in 7 of 8 mice.**

Conclusions: **Reduction in c-Met expression substantially inhibits both tumor growth and lymph node metastasis of PC3-LN4 cells in orthotopic nude mouse models. Therefore, targeting the c-Met signaling pathways may be important in controlling tumor growth and metastasis in human prostate cancers.**

Introduction

Cancer of the prostate is the most common cancer in men in North America and ranks second in cancer-related deaths in this population (1, 2). Despite efforts to promote early detection, >50% of patients have metastatic disease at diagnosis and initiation of treatment (3). As a result, metastases of hormone-refractory cells develop and ultimately lead to mortality. Currently, conventional treatments such as taxane-based chemotherapy (4, 5) are ineffective in treating patients with advanced disease. Thus, the identification of novel targets and new treatment regimens are critical for future control of advanced prostate cancer. Inhibitors of RPTKs,[2] often overexpressed in prostate cancer, may provide such targets for development of novel therapies.

One potential RPTK target in prostate cancer is c-Met. c-Met is expressed primarily in epithelial tissues (6). HGF, also known as SF, is the primary ligand of c-Met (6–8). Upon stimulation of HGF/SF, c-Met is tyrosine phosphorylated and initiates a cascade of signals that lead to activation of cellular behaviors such as mitogenesis, cell migration, matrix adhesion and invasion, angiogenesis, morphogenesis, survival, or combination of these events depending on the cell types stimulated. Mutations and aberrant overexpression of c-Met and HGF/SF have been correlated with disease progression and clinical outcome in a variety of cancers (9–14).

Aberrant signaling through c-Met has been associated with prostate tumorigenesis and progression in rodents and man. In the Nobel rat, overexpression of HGF and c-Met occurs during sex hormone-induced prostatic carcinogenesis (15). In the human disease, c-Met overexpression occurs as early as prostate intraepithelial neoplasia (16). Furthermore, strong correlative evidence also suggests a role for HGF/SF/c-Met signaling in the progression and metastasis of human prostate cancers. Analysis of prostate cancer surgical specimens demonstrates a direct

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[2] The abbreviations used are: RPTK, receptor protein kinase; HGF, hepatocyte growth factor; SF, scatter factor; VEGF, vascular endothelial growth factor; FBS, fetal bovine serum; MOI, multiplicity of infection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Erk, extracellular signal-regulated kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

association between increased c-Met expression and the formation of bone and lymph node metastases (17–19). More than 80% of all men who develop prostate cancer will develop metastatic disease in the bone, especially in the pelvis, femur and vertebrae and human bone stroma, a rich source of HGF/SF (20, 21). The possible role of HGF/SF in the preferential metastasis of prostate cancer to the bone was implicated by demonstrating the importance of this growth factor on the formation of prostate epithelial cell colonies on bone marrow stroma with epithelial cells from benign and malignant prostate tissues (22). HGF/SF and c-Met are also biomarkers of invasion of prostate cancer cells and progression of disease in latent and clinical prostate cancer, including prostatic intraepithelial neoplasia (23–29). Parr *et al.* (30) demonstrated that NK4, an HGF/SF variant, suppressed the HGF/SF-induced phosphorylation of paxillin, matrix adhesion, and invasion of prostate cancer cells. However, although substantial evidence implicates c-Met overexpression with prostate tumor progression, no studies have assessed the effects of reducing c-Met expression on tumor growth and metastasis.

To examine the role of c-Met in other tumor types in which it is overexpressed, the strategy of ribozyme-mediated-c-Met down-regulation has been used (31–33). In the glioblastoma cell lines U-373 (expressing c-Met only) and U-87 (expressing c-Met and HGF), Abounader *et al.* (32) demonstrated that stable ribozyme-expressing clones were reduced in c-Met expression by 95%. c-Met down-regulation in U-373 did not affect tumor growth or incidence. Reduction in tumor incidence and growth in the brain resulted only when an autocrine HGF/c-Met loop was present (32). Therapeutic treatment of orthotopically implanted U-87 tumors with adenovirus and/or liposomes containing the c-Met or HGF specific ribozyme increased the median survival of treated animals compared with untreated animals (31). Thus, in this model, c-Met down-regulation reduces tumorigenicity only when c-Met is activated by autocrine production of HGF. In two human breast cancer cell lines, MDA-MB 231 and MCF-7, ribozyme-mediated c-Met down-regulation significantly reduced HGF-induced migration and invasion (33) relative to a control (PU1) construct. Thus, expression of this c-Met ribozyme has proven successful in determining the biological effects of reducing c-Met expression in a highly specific manner. In this study, we report that reduction of c-Met expression by either *ex vivo* or *in vivo* infection with a defective adenovirus expressing a c-Met ribozyme of PC3P-LN4 prostate cancer cells, a metastatic variant of PC3P, inhibits tumorigenic growth and lymph node metastasis in orthotopic models in male nude mice. Previous studies have demonstrated that signaling through c-Met induces angiogenesis (34, 35). In accord with these results, we demonstrate that down-regulation of c-Met strongly down-regulates VEGF expression and, furthermore, that c-Src tyrosine kinase activity is, in part, responsible for reduced VEGF expression.

Materials and Methods

Cell Lines and Cell Culture Conditions. The PC-3 human prostate cancer cell line was originally obtained from the American Type Culture Collection (Manassas, VA). The PC-3M cell line was derived from a liver metastasis produced by parental PC-3 cells growing in the spleen of a nude mouse. PC-3M cells were implanted orthotopically into the prostate of nude mice, and after several cycles of *in vivo* selection through prostate and regional lymph nodes, the highly metastatic cell line to lymph node and distant organ, PC-3P-LN4, was isolated (36). The PC-3P-LN4 cell line was maintained as monolayer cultures in DMEM/F12 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FBS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, a 2-fold vitamin solution (Life Technologies, Inc.) in the absence of antibiotics. Cell cultures were maintained and incubated in 5% CO_2/95% air at 37°C. Cultures were free of *Mycoplasma* and the following murine viruses: reovirus type 3; pneumonia virus; K virus; Theiler's encephalitis virus; Sendai virus; minute virus; mouse adenovirus; mouse hepatitis virus; lymphocytic choriomeningitis virus; ectromelia virus; and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD).

Animals. Male athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The mice were housed and maintained in specific pathogen-free conditions. The facilities were approved by the American Association for Accreditation of Laboratory Animal Care and meet all current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. The mice were used in accordance with Institutional guidelines when they were 8–12 weeks old.

Adenovirus Ribozyme Construct Targeting Human c-Met. A plasmid expressing the c-Met ribozyme was constructed by Abounader *et al.* (32). Briefly, pZeoU1EcoSpe parent vector was derived from wild-type U1snRNA, which is an essential component of the spliceosome complex and is stable and abundant in the nucleus. The U1 promoter was cloned in a *Bam*HI site of a modified pZeo-EcoSpe vector, which is zeocin resistant. A hammerhead ribozyme that recognizes and cleaves the GUC sequence was used. The complementary pair of ribozyme oligonucleotides that span between 540 and 560 with a GUC sequence at position 560 of human c-Met was synthesized, annealed at 40°C, and ligated into pU1 at the *Eco*RI and *Spe*I site to create the vector. As a control, an irrelevant sequence was also cloned into pU1 between *Eco*RI and *Spe*I sites and was designated (Ad PU1).

Replication-defective adenoviral vectors of the ribozyme expressing (Ad-c-Met) and control (Ad-PU1) constructs were constructed as previously described by Abounder *et al.* (32), and purified viruses were a generous gift of Dr. John Laterra (Kennedy Krieger Research Institute). For viral stock, adenoviruses were infected into 293 embryonic kidney cells. Cells were harvested 36–40 h after infection, pelleted, resuspended in PBS, and lysed by three freeze-thaw cycles. Cell debris was removed, and resulting cellular lysates were subjected to double CsCl centrifugation. Concentrated virus was dialyzed, aliquoted, and stored at −80°C. Adenovirus titer represented as optical particles units was determined by optical absorbance as described previously (28). Infectious viral titers were determined by quick cytopathic effect assays. Viral particles/ml were calculated by using the following formula: [(number of cells/well) × (dilution with cytopathic effect, 10^4) × (10 virus/cell)]/0.3 ml viral

sample/well. For infection of PC3-LN4 cells, cultures at 70% confluency were infected with at the indicated MOI. MOI was determined by plaque forming units in 293 s cells after serial dilution.

Immunoblotting. Cells (1×10^6) were plated in monolayer on a 10-cm dish and infected with either Ad-PU-1 or Ad-c-Met for 24 h. Cells were washed twice with Ca^{2+}, Mg^{2+}-free ice-cold PBS [135 mM NaCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4 (pH 7.4), and 2.5 mM KCl] and lysed on ice in lysis buffer [50 mM HEPES (pH 7.0), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM Na PP_i, 10% glycerol, 1% Triton X-100, and 1 mM Na_2VO_4] plus 1 tablet/10 ml lysis buffer consisting of Complete Mini, EDTA-free protease inhibitor mixture tablets (Roche) for 10 min before clarification by centrifugation at 15,000 rpm at 4°C for 15 min. Protein concentrations were quantitated by using the Detergent-Compatible Protein Assay system (Bio-Rad, Hercules, CA) and absorbance measured by a spectrophotometer at 750 nm. Whole cell lysates were boiled for 5 min then loaded in Laemelli's sample buffer (2% SDS, 5% mercaptoethanol, 125 mM Tris (pH 6.8), 1 mM EDTA, 10% glycerol, and 0.02% bromphenol blue] and resolved by SDS-PAGE on an 8% polyacrylamide gel. Proteins were transferred to NitroPure nitrocellulose membranes (Fisher Scientific). Membranes were blocked for 1 h in blocking buffer (5% dry milk; Kroger, Houston, TX) in Tris-buffered saline pH 7.5 (TBS-T, 100 mM NaCl, 50 mM Tris, and 0.05% Tween 20). Blots were probed with anti-c-Met (clone C-28; Santa Cruz Biotechnology, Santa Cruz, CA), anti-actin (clone AC-15; Sigma-Aldrich, St. Louis, MO), anti-GAPDH antibody (Chemi-Con, Temecula, CA) anti-Src (clone 327; Oncogene Research Products, Cambridge, MA) mouse monoclonal antibodies, and/or anti-Erk1/2 (Ab-2; Oncogene Research Products), anti-phospho-p44/42 mitogen-activated protein kinase (Thr^{202}/Tyr^{204}; Cell Signaling Technology, Beverly, MA), anti-Akt (Cell Signaling Technology), and anti-phospho-Akt (Ser^{473}; Cell Signaling Technology) rabbit antisera as indicated. Antibodies were diluted in TBST [Tris-buffered saline-0.1% Tween 20 (v/v)] with 5% dried milk. Peroxidase-conjugated secondary antisera, goat antimouse antisera (Bio-Rad), or goat antirabbit (Bio-Rad) were used to detect the respective primary antibodies. Immunoreactive proteins were visualized with Chemiluminescence Reagent Plus detection system (NEN, Boston, MA), followed by and exposing the membrane to Kodak Biomax MR film (Amersham, Piscataway, NJ). Bands were scanned using a Hewlett-Packard ScanJet scanner and quantitated using Scion Image. The densitometry readings of the chemiluminescent bands were normalized to GAPDH or actin expression. Control lanes were normalized to 1.0.

Src Immune Complex Kinase Assay. Cells were rinsed with ice-cold PBS then detergent lysates were made as described above. Cells were homogenized and clarified by centrifugation at $10,000 \times g$. Cell lysates (250 μg protein) were reacted for 2 h with the Src monoclonal antibody 327 (Oncogene Research Products). Immune complex kinase assays were performed as previously described by Windham *et al.* (37). Briefly, immune complexes were formed by the addition of 6 μg of rabbit antimouse IgG (Organon Teknika, Durham, NC) for 1 h, followed by 50 μl of 10% (v/v) formalin-fixed Pansorbin (*Staphylococcus aureus*, Cowan strain; Calbiochem, La Jolla, CA) for 30 min. Pellets were then washed three times in a buffer consisting of 0.1% Triton X-100, 150 mM NaCl, and 10 mM sodium phosphate. Reactions were initiated at 22°C by the addition to each sample of 10 μCi of [γ^{32}P]ATP, 10 mM Mg^{2+}, and 100 μM sodium orthovanadate in 20 mM HEPES buffer. To analyze phosphorylation of an exogenous substrate, 10 μg of rabbit muscle enolase (Sigma-Aldrich) were added to the reaction buffer. After 10 min, reactions were terminated by the addition of SDS sample buffer. Proteins were separated by SDS-PAGE on 8% polyacrylamide gels, and radioactive proteins were detected by autoradiography.

Cell Proliferation. The MTT assay was used to estimate growth rates of cells. PC3-LN4 cells (4×10^5) were plated in monolayer on a 10-cm dish and infected with Ad-Pu1 or Ad-c-Met at MOIs of 25 and 50. Cells were trypsinized, and viable cells were counted using a hemacytometer after trypan blue staining. One thousand cells for each line were plated in triplicate each on a 96-well plate. Every 24 h, 50 μl of MTT (Sigma-Aldrich) were added to each well. After 2 h of incubation, all media were removed, and the cells were solubilized with the addition of 200 μl of DMSO (Fisher Scientific). The absorbance readings were performed on a spectrophotometer at 750 nm. The optical densities readings were divided by the reading at time 0 to obtain the percent increase in growth and plotted on a log scale *versus* time.

Migration and Invasion Assays. Migration assays were performed using a modified Boyden chamber system with a filter containing 8-μm pores (Control Inserts; Fisher Scientific). Invasion chambers (Biocoat Matrigel Invasion Chambers; Fisher Scientific) are identical to the migration chambers, with the addition of Matrigel layered on the top of the insert. For both assays, the bottom chamber was filled with 500 μl of conditioned media and then the insert was placed in the conditioned media. Conditioned media were obtained by growing NIH 3T3 fibroblasts to 80% confluency at 72 h in DMEM:Ham's F-12 + 10% FBS. After 72 h, the media were removed from the cells and filter sterilized. For the migration and invasion assays, equal numbers of cells in a volume of 500 μl for each cell line were suspended in their growing media and placed in the top chamber. The chamber was placed at 37°C for the length of the assay. To assess the cells that had migrated, the cells on the top of the filter were removed with a cotton swab. The cells that migrated through and adhered to the bottom of the filter were fixed and stained with the Hema 3 Stain Set (Fisher Scientific) according to the manufacturer's instructions. The stained cells on each insert were counted in five ×200 fields and averaged to give n = 1/insert. The sample size was therefore the number of inserts.

VEGF Expression. To determine expression of VEGF protein, conditioned medium from PC3-LN4 control and infected cells were harvested after 48 h from cells growing in 1% FBS-containing medium. The VEGF concentration was determined using an ELISA kit for human VEGF (Biosource) as we have previously reported (38, 39) [the ELISA kit recognizes the VEGF121 and VEGF165 isoforms of the protein (40)].

***Ex Vivo* Infection and Orthotopic Implantation of PC3 LN-4 Cells.** Tumor cells (1×10^6) were infected for 24 h with either Ad-PU1 or Ad-c-Met at an MOI of 25. Cells were harvested after a brief exposure to 0.25% trypsin:0.1% EDTA

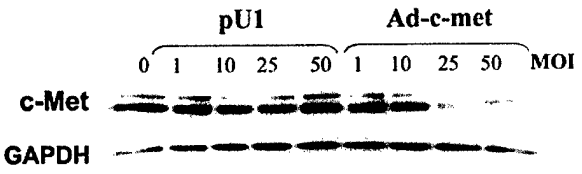


Fig. 1 Effects of Ad-PU1 and Ad-c-Met infection on c-Met expression in PC3-LN 4 cells. Cells were mock infected or infected with the indicated MOI of adenovirus, and immunoblotting was performed for c-Met expression as described in "Materials and Methods."

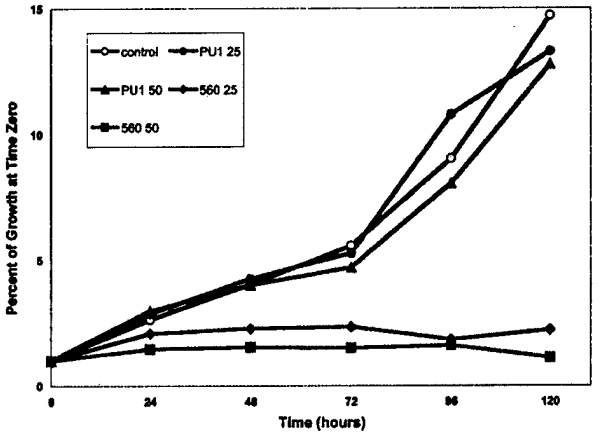


Fig. 2 Effects of infection with Ad-PU1 and Ad-c-Met on proliferation of PC3-LN4 cells. Cells (4×10^5) were seeded in 10-cm tissue culture dishes and mock infected or infected with adenovirus at the indicated MOI. Proliferation was estimated by the incorporation of MTT, as described in "Materials and Methods."

solution (w/v). After dislodging cells, the cell suspensions in culture media were pipetted to obtain single-cell suspensions and counted for cell viability in trypan blue with a hemacytometer. A total of 2.5×10^6 cells/group was counted, centrifuged, and resuspended in sterile Ca^{2+}- and Mg^{2+}-free HBSS at 5×10^5 cells/50 μl. Orthotopic injection was done as described previously (36). In brief, nude mice were anesthetized with Nembutal (Abbott Laboratories, North Chicago, IL) and placed in a supine position. A low midline incision was made, and the prostate was exposed. Fifty μl of HBSS containing 5×10^5 cells were injected into a lateral lobe of this prostate. The wound was closed with surgical metal clips. A total of 10 mice/group was injected.

Gene Therapy Studies. Subconfluent cultures of PC3P-LN4 cells were harvested by trypsinization. Cells were counted and resuspended in Ca^{2+}- and Mg^{2+}-free HBSS at 50,000 cells/50 μl. Cells were then implanted in the prostate of nude mice as described above, and the wound was closed with surgical metal clips. Tumors were treated at 7 and 14 days after implantation. Tumors were exposed by the same procedure by low midline incision and exposure of the prostate. Tumors were injected with PBS or 1.5×10^9 plaque forming units of Ad-c-Met or Ad-PU1, each in a total volume of 20 μl. Tumors and lymph nodes were harvested 14 days after the final gene intratumoral injection. Each experimental condition was performed on groups of 8 mice.

Necropsy Procedures and Histological Studies. The mice were euthanized at 4 weeks after tumor cell injection, and body weights were determined. Primary tumors in the prostate were excised, measured, and weighed. Specimens were formalin-fixed and paraffin-embedded for H&E staining. Macroscopically enlarged lymph nodes were harvested, and the presence of metastatic disease was confirmed by histology.

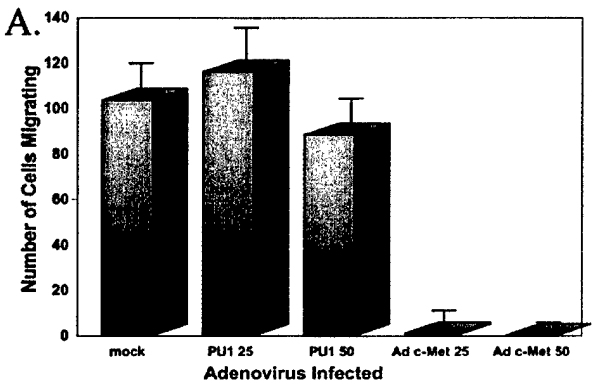
Statistical Analyses. Tumor incidence and incidence of lymph node metastasis (χ^2 test), tumor weight (Mann-Whitney test), total and phosphorylated c-Met protein expression (Student's *t* test), and cellular proliferation (Student's *t* test) were compared statistically.

Results

Reduction of c-Met Protein Levels in PC3-LN4 Cells by Ad-c-Met Infection. PC3-LN4 human prostate cancer cells express high c-Met levels, with constitutive tyrosine phosphorylation and high intrinsic tyrosine kinase activity, characteristic of cell lines with activated c-Met. Although c-Met can be activated by mutation in the tyrosine kinase domain (12, 13), no mutations in this region were observed in c-Met in PC3-LN4 cells (data not shown). To examine the effects of c-Met down-regulation, PC3-LN4 cells were mock infected or infected with PU1 and Ad c-Met adenoviruses. As shown in Fig. 1, infection with pU1 (control) virus did not change the expression of c-Met at MOIs as high as 50. In contrast, infection with MOIs of 25 and 50 decreased c-Met expression by 95% relative to PU1 infection at similar MOIs and corrected for loading by GAPDH expression.

Inhibition of Cellular Proliferation of PC3-LN4 Cells by Ad-c-Met Infection. To determine the *in vitro* growth rates of c-Met down-regulated cells, MTT analysis was performed at 24, 48, 96, and 120 h after infection. The results are shown in Fig. 2. Infection with pU1/control adenovirus did not affect cellular proliferation of PC3-LN4 cells, but c-Met ribozyme adenovirus significantly inhibited the cellular proliferation of PC3-LN4 cells. Growth inhibition rates (percentage) at 24, 48, 72, 96, and 120 h were 33.0 ± 7.3, 52.5 ± 3.0, 64.5 ± 5.2, 82.8 ± 0.9, and 87.2 ± 0.8 with 25 MOI and 44.6 ± 2.7, 62.1 ± 4.4, 73.1 ± 1.0, 82.3 ± 1.4, and 92.4 ± 0.8 with 50 MOI, respectively ($P < 0.01$, paired Student's *t* test). There was no significant difference in inhibitory effect of cellular proliferation between the two different MOIs. These results suggest that c-Met expression is important to proliferation and/or survival of PC3-LN4 cells.

Suppression of Migration and Invasion by Ad-c-Met Infection. One of the principal functions of c-Met is promotion of migration, a process also important in metastasis. To examine the effects on migration of reduced c-Met expression in PC3-LN4 cells, single cells were seeded in the upper chamber of a standard two-chamber migration assay (Boyden chamber), and cells migrating to the bottom of the insert were counted as described in "Materials and Methods." As shown in Fig. 3*A*, PC3-LN4 cells were substantially reduced in migratory capacity when infected with Ad c-Met but not Ad-PU1, with almost no cells migrating upon infection with an MOI of 50 (a 97%



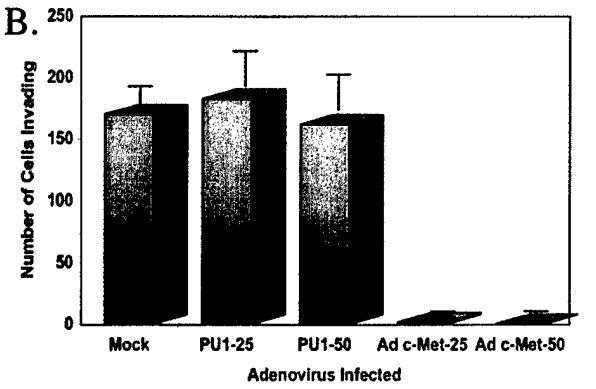


Fig. 3 Effects of infection of Ad-PU1 and Ad-c-Met on migratory and invasive potentials of PC3-LN4 cells. Migration and Invasion assays were performed using a modified Boyden chamber system with a filter containing 8-μm pores. NIH 3T3 conditioned medium was used as a chemoattractant in the bottom chamber, and equal numbers of infected cells or mock-infected cells were placed on the top chamber and allowed to grow for 72 h. Cells that migrated to the bottom of the insert were counted as described in "Materials and Methods." *A*, migration assays in which Boyden chambers were uncoated; *B*, invasion assays in which inserts were coated with Matrigel.

decrease to an average of <1 cell ± 0.31, $P < 0.01$, unpaired Student's *t* test). Parallel assays in which cells on the top of the insert were stained with trypan blue indicated that the cells were viable. To assess the ability of cells to invade through an extracellular matrix-like environment, Boyden chambers were used in which inserts were coated with Matrigel, and invasion assays were also performed as described in "Materials and Methods." The results are shown in Fig. 3*B*. Very similar results were seen to that of the migration assay, with no significant effect of the PU1 [control virus on invasion and an almost complete inhibition of invasive capacity at an MOI of 50 of Ad c-Met (99% decrease in cells invading to an average of <1 cell ± 0.11, $P < 0.001$, unpaired Student's *t* test)]. Thus, reduction of c-Met corresponded with reduced biological functions resulting from c-Met signaling.

Tumorigenicity in the Prostate after *ex Vivo* Infection of Ad-c-Met. As c-Met down-regulation decreased the *in vitro* growth rates, migration and invasion activity, *in vivo* growth rates, and metastatic behavior were analyzed. Cells were infected with adenovirus or mock infected and were implanted orthotopically into male nude mice as described in "Materials and Methods." Four weeks after injection, prostates were harvested and analyzed. In mock-infected PC3-LN4 cells 88.9% (8 of 9) mice developed tumors. Similarly, 88.9% (8 of 9) mice formed prostate tumors with pU1/control virus-infected PC3-LN4 cells (Table 1). There was no significant difference in tumor weight between mock-infected PC3-LN4-injected group (median: 0.58g) and pU1/50 adenovirus-infected PC3-LN4-injected group (median: 0.47g; $P > 0.05$, Mann-Whitney test). In mock-infected and the Ad-pU1/50 control virus-infected group, lymph nodes of tumor-bearing mice were enlarged and macroscopically confirmed of their tumor involvement by H/E staining (Figs. 4 and 5). In contrast, infection with Ad-c-Met significantly reduced tumor incidence and tumor growth with 10% of animals (1 of 10) developing tumors, and the one macroscopically detectable tumor was very small (0.07g) with largely normal histology by H&E staining (Fig. 5*D*). There were no mice with detectable lymph node metastases (Fig. 4*C*). Statistical comparison in tumor weight was inappropriate because of the low tumorigenicity induced by Ad-c-Met-infected cells.

Table 1 Tumorigenicity and lymph node metastases induced by PC3-LN4 cells after *ex vivo* infection of adenovirus or mock infection

Group[a]	Tumor incidence[b]	Tumor size (g) Median (range)	Lymph node involvement[c]
Mock	8/8	0.58 (0.31–1.01)	8/8
Ad-Pu-1	9/9	0.47 (0.01–2.45)	9/9
Ad-c-Met	1/10	0.07	0/1

[a] PC3-LN4 cells were mock infected, or infected with the indicated adenovirus at an MOI of 50, and 5×10^5 cells were injected into the prostates of nude mice, as described in "Materials and Methods."

[b] Number of mice with tumors/number of mice surviving surgery for prostate tumor cell implantation.

[c] Number of mice with lymph node metastases/number of mice with tumors.

Effects of Intraprostatic Injection of Adenoviruses into Preestablished PC3-LN4 Tumors. To determine whether reduced c-Met expression could affect the growth of PC3-LN4 cells growing in the prostate, gene therapy experiments were performed by allowing the tumor cells to grow in the prostate for 7 days, then injecting Ad-c-Met, Ad-PU1, or sham-injecting mice in the prostate 7 and 14 days later as described in "Materials and Methods." The results of these experiments are shown in Table 2. In 7 of 8 mice infected with Ad-PU1 and 8 of 8 sham-injected mice, tumors developed in the prostate. In contrast, only 1 of 8 mice injected with Ad-c-Met developed a tumor and that tumor was substantially smaller than tumors forming in control mice (Table 2). These results suggest that c-Met is performing an important survival function in PC3-LN4-induced tumors, and c-Met reduction is incompatible with development of macroscopic tumors.

Decreased c-Met Decreases VEGF Expression and Src Kinase Activity. Ample evidence has implicated c-Met signaling in angiogenesis and especially expression of VEGF (34, 35, 41, 42). To determine whether decreased VEGF expression along with decreased proliferation rates might explain, in part, the growth inhibition of tumors after decreased c-Met expres-

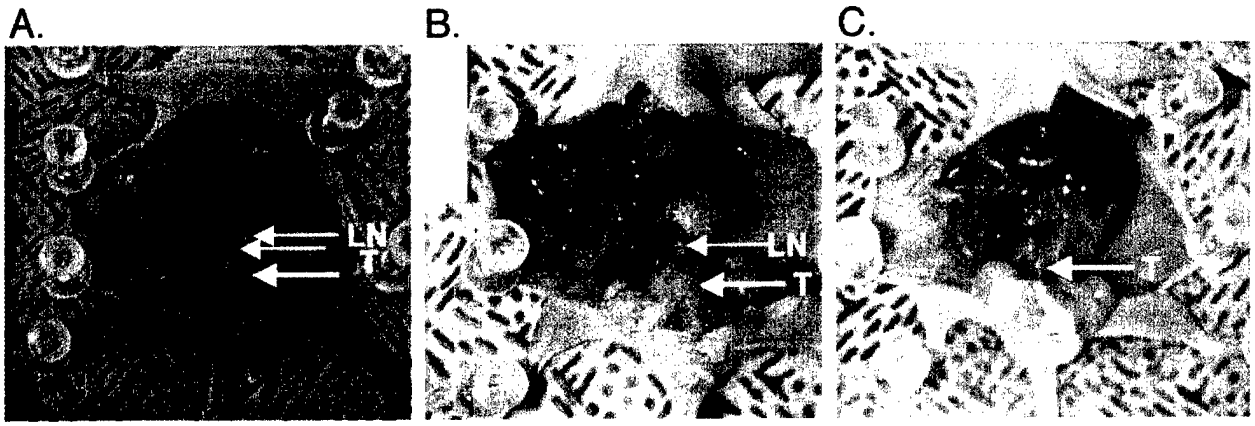


Fig. 4 Tumor formation in nude mice following: mock infection (*A*); *ex vivo* infection with Ad-PU-1 control adenovirus (*B*); or *ex vivo* infection with Ad-c-Met (*C*). PC3 LN-4 cells were mock infected or infected with given adenoviruses at an MOI of 50, after which cells were implanted into the prostate of nude mice as described in "Materials and Methods." Mice were killed 4 weeks later and examined for presence of tumors. *C* displays the only mouse implanted with PC3 LN-4 Ad c-Met-infected cells with macroscopic evidence of tumor.

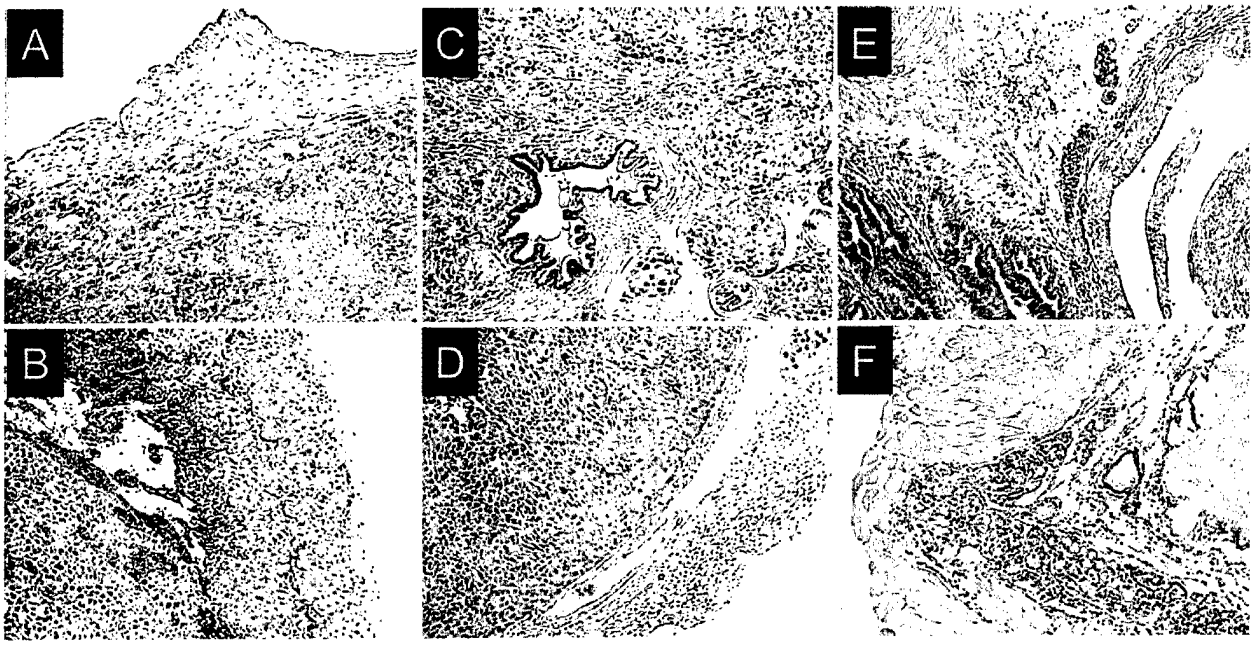


Fig. 5 H&E staining of nude mouse prostate tumors after orthotopic injection of mock, Ad-PU 1 control virus, or Ad-c-Met-infected PC3-LN4 cells. Primary tumor (*A*) and lymph node metastasis (*B*) from mice with mock-infected cells; primary tumor (*C*) and lymph node metastasis (*D*) from PU-1-infected cell; H&E of possible primary tumor from Ad-c-Met-infected cells (*D*); normal prostate (*E*).

sion, we examined VEGF expression in the media of PC3-LN4 cells 48 h after infection with ribozyme-expressing (Ad-c-Met) or control (PU-1) virus. The results are shown in Fig. 6. Significant reduction in VEGF expression (per 10^6 cells as measured by ELISA) was observed in PC3-LN4 cells in Ad-c-Met-infected cells relative to Ad-PU1 (control)-infected cells.

To begin to examine signaling pathways that might be affected by c-Met expression and are important to VEGF expression, we determined the status of Erk1/2, Akt, and Src after down-regulation of c-Met by Ad-c-Met. The activity of Src, (Src autophosphorylation and phosphorylation of the exogenous substrate enolase) in the immune complex kinase assay is reduced in PC3-LN4 cells infected with Ad-c-Met, with >50% reduction observed in autophosphorylation at an MOI of 25 and >80% reduction of autophosphorylation observed at an MOI of 50, relative to uninfected controls (Fig. 7). Phosphorylation of the exogenous substrate enolase was also reduced relative to uninfected controls in a dose-dependent manner, although to a lesser extent than autophosphorylation. Only a small reduction in Src activity was observed upon infection with high MOIs of the

Table 2 Tumorigenicity and lymph node metastases after mock or intraprostatic infection of adenovirus into nude mice injected intraprostatically with PC3-LN4 cells

Group[a]	Tumorigenicity[b]	Tumor size (g) Median (range)	Lymph node involvement[c]
Sham	8/8	0.66 (0.48–0.99)	8/8
Ad-PU1 (50MOI)	7/8	0.48 (0.4–0.59)	7/7
Ad-c-Met (25MOI)	1/8	0.04	0/1
Ad-c-Met (50MOI)	1/8	0.08	0/1

[a] PC3-LN4 cells (5×10^5) were injected into the prostates of nude mice as described and allowed to grow for 7 days, after which indicated adenovirus (or sham injection) was delivered 7 and 14 days after tumor cell implantation.

[b] Number of mice with tumors/number of mice injected.

[c] Number of mice with lymph node metastases/number of mice with tumors.

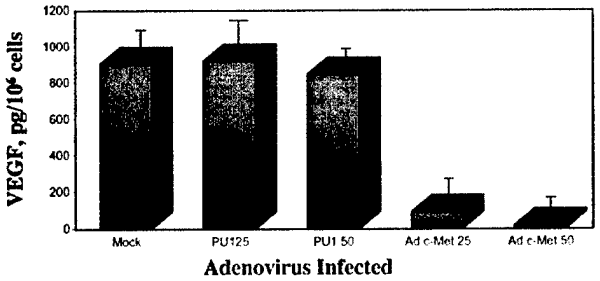


Fig. 6 Expression of VEGF in PC3 Ln-4 cells infected with Ad-c-Met or Ad-PU1. Conditioned medium from PC3-LN4 control and infected cells was harvested after 48 h from cells growing in 1% FBS-containing medium, and VEGF expression was determine by ELISA, as described in "Materials and Methods." To account for differences in growth rates, VEGF expression is expressed as pg VEGF/10^6 cells.

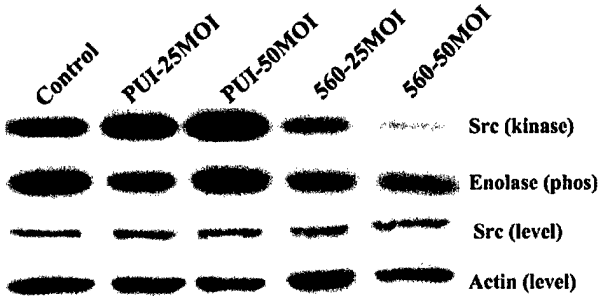


Fig. 7 Expression and activity of c-Src in mock, Ad-c-Met, and Ad-Pu1-infected PC3 LN-4 cells. Cells were mock-infected or infected with adenoviruses at indicated MOIs. To determine Src activity, cell lysates were immunoprecipitated with the Src-specific antibody mAb 327, and the immune complex kinase assay was performed with addition of enolase as an exogenous substrate as described in "Materials and Methods." To determine expression of Src, immunoblotting with mAb 327 was performed as described previously.

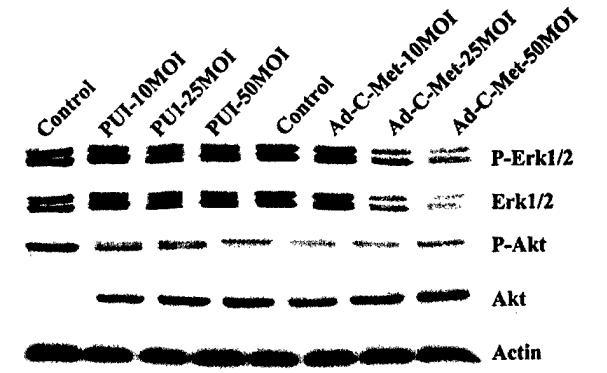


Fig. 8 Expression of phosphorylated and total Erk1/2 and Akt in mock, Ad-c-Met, and Ad-PU-1-infected PC3 LN-4 cells. Cell lysates were subject to SDS-PAGE, electrotransferred to NitroPure nitrocellulose membranes, and incubated with phospho-Akt-specific or phospho-Erk1/2-specific antibodies. Blots were stripped and reprobed with antibodies recognizing phosphorylated and nonphosphorylated Akt or Erk 1/2 as previously described in "Materials and Methods."

control virus, and no obvious changes in Src expression were observed at the MOIs used for control or ribozyme-expressing virus (Fig. 7). These results demonstrate that down-regulation of c-Met down-regulates Src activity. In contrast, adenovirus infection (*i.e.*, either Ad-c-Met or Ad-PU1) appeared to increase expression of Akt, but no differences in phosphorylation were observed between control (Ad-PU1) and ribozyme-expressing (Ad-c-Met) viruses (Fig. 8). Erk1/2 expression appeared somewhat decreased by infection with Ad-c-Met, but this decrease was <2-fold and likely reflects the slower proliferation rate of these cells. Thus, decreased VEGF expression observed after c-Met down-regulation correlates with decreased c-Src activity.

Discussion

Despite surgical removal of organ-confined lesions, the biological unpredictability of prostate cancer does not guarantee patient survival. As with most solid tumors, patients that succumb to prostate cancer almost invariably do so because of metastatic spread of the disease. Metastasis of androgen-independent prostate cancer to the bone causes devastating symptoms such as intractable bone pain, nerve compression and pathological fractures, and at this stage, tumors rarely respond to conventional chemotherapies (4, 5). Therefore, development of new treatment modalities inhibiting progression and metastasis of the disease are clearly needed. Inhibition of receptor protein tyrosine kinases has shown considerable promise in the treatment of some tumors. In prostate tumors, aberrant expression of RPTKs is relatively common. For example, members of the ErbB family, specifically HER1, HER2, HER3 (43, 44) but not HER4 (45), are frequently overexpressed. This overexpression is associated with androgen-independent growth of prostate tumor cells, suggesting that overexpression of this gene family promotes tumor progression. One of the most consistently overexpressed RPTKs in prostate cancer is c-Met. Analysis of clinical specimens has linked c-Met overexpression to prostate tumor progression (16–18, 23, 24, 26); however, no studies have examined the cause/effect relationship between c-Met tumor growth and metastasis in an orthotopic model.

In this study, we used PC3-LN4 cells, a variant of PC3-P

cells that by passage through nude mice is increased in metastatic potential to the lymph nodes. Reduction of c-Met expression was achieved by an adenovirus expressing a highly specific c-Met ribozyme as described by Abounader *et al.* (32). The approach of using adenoviruses expressing ribozymes has been highly successful in specifically reducing the expression of diverse gene products such as HER2/neu (46) and Bcl-2 (47). The selectivity of this approach ensures that c-Met alone is targeted, thus allowing specific conclusions to be derived with respect to c-Met function. In our studies, >90% reduction of c-Met expression resulted from adenovirus infection at an MOI of 50, and inhibition of c-Met alone was sufficient to affect biological functions of prostate tumor cells. In colon tumor cells, we have demonstrated that reductions of only ~30% in c-Met expression are sufficient to decrease the ability to form tumors in the livers of nude mice (48). In contrast, in glioblastoma cells, Abounader *et al.* (32) required inhibition of both c-Met and its ligand, HGF, to affect tumor growth. Thus, overexpression alone in PC3P-LN4 cells appears to be sufficient to activate signaling pathways important to the biological properties of these cells. Furthermore, similar *in vitro* effects were observed with PC3P cells (data not shown), suggesting that c-Met overexpression is not important only to selected variants of high metastatic potential.

The main focus of this study was to examine the effect of c-Met down-regulation in tumor cells on *in vivo* growth and metastasis of tumor cells. Therefore, we compared the tumor growth in orthotopic site and development of metastases to the regional lymph nodes. We used two approaches for these studies: *ex vivo* infection of adenoviruses into PC3-LN4P cells followed by orthotopic implantation and orthotopic implantation of tumor cells followed by *in vivo* injection of adenoviruses into the prostate. Down-regulation of c-Met expression after *ex vivo* infection significantly reduced tumor formation or delayed growth in the prostate. Strikingly, *in vivo* delivery of the ribozyme by intraprostatic injection of Ad-c-Met led to highly similar results. Thus, overexpression of c-Met in prostate tumor cells alone appears to play an important role in the ability of these cells to grow. As c-Met can be activated by HGF/SF in the stromal cells of the prostate (49), these results suggest that inhibition of c-Met in the tumor cells may also inhibit receptor activation induced by HGF in the tumor microenvironment. *In vitro* studies also demonstrated that down-regulation of c-Met reduced not only proliferation rates but also cellular migration and cellular invasion and VEGF expression, suggesting that c-Met may play multiple roles in prostate tumor progression.

Although a large number of signaling cascades are induced upon c-Met activation [reviewed by Furge *et al.* (50) and Maulik *et al.* (51)], we demonstrated that decreased c-Src activity, but not Akt or Erk1/2 phosphorylation, occurred upon c-Met down-regulation. This result is consistent with Src constitutively docking with c-Met when c-Met is overexpressed and therefore activated. Strong biochemical (52) and genetic evidence (53) support a role for Src in several c-Met-mediated functions, including migration and proliferation, and activated c-Met requires functional Src for tumorigenic growth of NIH 3T3 cells in nude mice (54). As Src is involved in a number of survival pathways [reviewed by Schlessinger (55)], it is likely that Src activation also contributes to critical survival pathways in prostate tumor growth and/or progression. However, the multifunctional roles of c-Met in activating signal transduction pathways suggest that additional pathways important to tumor progression may be down-regulated as well and that decreased Src activity may be only one of the factors contributing to decreased VEGF expression. Nevertheless, we have demonstrated previously with this model system that ectopic expression of PTEN inhibited the development of lymph node metastases but not that of primary tumors (56). Furthermore, vascularization of tumors was unchanged (56). Ectopic expression of PTEN does not affect c-Src activity (G. E. Gallick, unpublished observations), suggesting that promotion of angiogenesis by c-Met through Src activation may be early event in prostate tumor progression. Although it is difficult to determine whether the down-regulation of c-Met significantly reduced metastatic potential of the PC3-LN4 cells because of low tumorigenicity and low tumor burden (delayed growth), the *in vitro* data showing reduced migration and invasion activity suggests that suppression of metastatic activity of the cells by down-regulation of c-Met is highly probable. Thus, altering gene expression in prostate tumor cell lines and examining the effects in orthotopic models is providing insights as to not only what roles specific gene products play in tumor growth and progression but also where in progression of the disease these alterations are important. These results have potential important therapeutic implications as protein tyrosine kinase inhibitors continue to reach the clinic. Understanding which pathways are required for various stage of prostate tumor progression should lead ultimately to understanding which inhibitors and combinations thereof may be of efficacy in different state tumors.

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